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Agency

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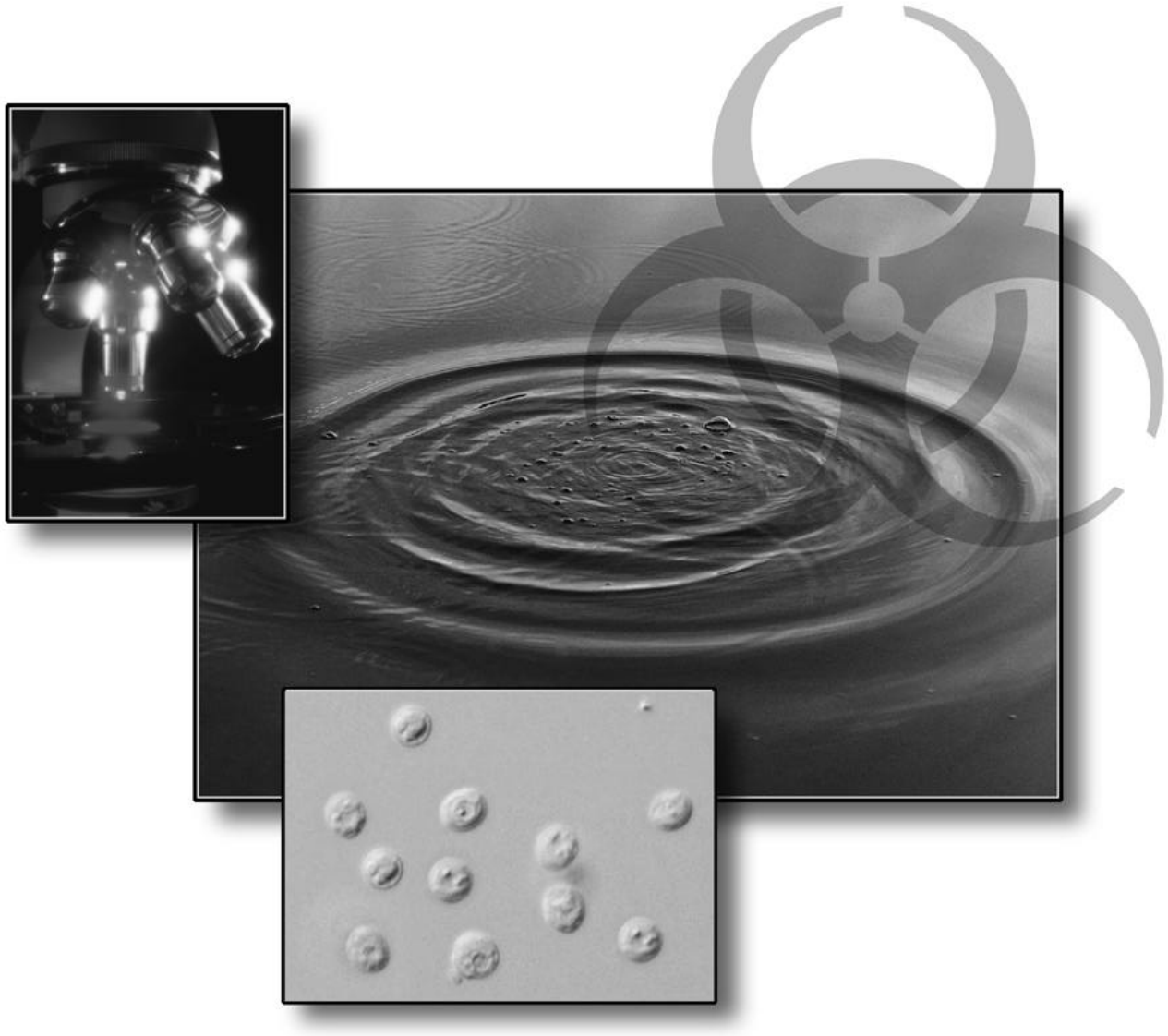
Method 1622: *Cryptosporidium* in Water by Filtration/IMS/FA



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Acknowledgments

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Mike Arrowood, Centers for Disease Control, Division of Parasitic Diseases (MS-F13), 4770 Buford Highway, N.E., Atlanta, GA 30341-3724, USA
Phil Berger, Office of Groundwater and Drinking Water, U.S. Environmental Protection Agency, 401 M Street, S.W., Washington, DC 20460, USA
Jennifer Clancy, Clancy Environmental Consultants, Inc., P.O. Box 314, St. Albans, VT 05478, USA
Kevin Connell, DynCorp I&ET, 6101 Stevenson Avenue, Alexandria, VA 22314, USA
Ricardo DeLeon, Metropolitan Water District of Southern California, 700 Moreno Avenue, LaVerne, CA 91760, USA
Colin Fricker, Thames Water Utilities, Manor Farm Road, Reading, Berkshire, RG2 0JN, England
Carrie Hancock, CH Diagnostic & Consulting Service, Inc., 214 S.E. Nineteenth Street, Loveland, CO 80537, USA
Stephanie Harris, Region 10 Laboratory, U.S. Environmental Protection Agency, 7411 Beach Drive East, Port Orchard, WA 98366, USA
Dale Rushneck, Interface, Inc., 3194 Worthington Avenue, Fort Collins, CO 80526, USA
Frank Schaefer III, National Exposure Research Laboratory, U.S. Environmental Protection Agency, 26 W. Martin Luther King Drive, Cincinnati, OH 45268-1320, USA
Steve Schaub, Health and Ecological Criteria Division (4304), Office of Science and Technology, U.S. Environmental Protection Agency, 401 M Street, S.W., Washington, DC 20460, USA
Huw Smith, Department of Bacteriology, Scottish Parasite Diagnostic Laboratory, Stobhill NHS Trust, Springburn, Glasgow, G21 3UW, Scotland
Timothy Straub, Lockheed Martin, 7411 Beach Drive East, Port Orchard, WA 98366, USA
William A. Telliard, Office of Science and Technology, U.S. Environmental Protection Agency, 401 M Street, S.W., Washington, DC 20460, USA

Disclaimer

This method has been reviewed by the U.S. EPA Office of Water and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Questions regarding this method or its application should be addressed to:

William A. Telliard
U.S. EPA Office of Water
Analytical Methods Staff
Mail Code 4303
Washington, DC 20460
Phone: 202/260-7120
Fax: 202/260-7185 /a

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Introduction

To support future regulation of protozoa in drinking water, the Safe Drinking Water Act Amendments of 1996 require U.S. Environmental Protection Agency (U.S. EPA) to evaluate the risk to public health posed by drinking water contaminants, including waterborne parasites, such as *Cryptosporidium*. To implement these requirements, EPA must accurately assess *Cryptosporidium* occurrence in raw surface waters used as source waters for drinking water treatment plants, determine drinking water treatment and disinfection process needs, and set meaningful protozoa standards for drinking water. Method 1622 was developed to detect *Cryptosporidium* reliably in raw surface water.

The U.S. EPA initiated an effort in 1996 to identify new and innovative technologies for protozoan monitoring and analysis. After evaluating potential alternatives to current methods through literature searches, discussions with research and commercial laboratories, and meetings with experts, the U.S. EPA Office of Water developed an initial draft of Method 1622 in December 1996. The draft method was revised in January, May, and December 1997, based on comments from experts, multiple in-laboratory peer reviews, and two single-laboratory validation studies. A interlaboratory validation study involving 12 laboratories and 12 raw surface water matrices was conducted in August 1998 to assess the method's recovery and precision for *Cryptosporidium* in reagent water and raw surface water. The method is valid for use in these matrices.

Method 1622 is a performance-based method applicable to the determination of *Cryptosporidium* in aqueous matrices. Method 1622 requires filtration, immunomagnetic separation of the oocysts from the material captured, and an immunofluorescence assay for determination of oocyst concentrations, with confirmation through 4',6-diamidino-2-phenylindole (DAPI) staining and differential interference contrast (D.I.C.) microscopy.

The single-laboratory and interlaboratory validations of Method 1622 used the Pall Gelman Laboratory capsule filtration procedure and Dynal immunomagnetic separation (IMS) procedures described in this method. However, alternate procedures are available for filtration and IMS. Alternate non-determinative techniques are allowed, provided that required quality control (QC) tests are performed and all QC acceptance criteria in this method are met.

Alternate filtration procedures to the validated Pall Gelman Laboratory capsule filter procedure include, but are not limited to, the following:

- Corning membrane disk filtration
- Corning capsule filtration
- ImmuCell vortex flow filtration
- Modified blood-cell separation technology developed by the Marshfield Medical Research Foundation
- Continuous-operation water centrifuge developed by the Johns Hopkins University

Alternate IMS procedures to the validated Dynal IMS procedure include, but are not limited to, the following:

- ImmuCell IMS
- Hach IMS

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Method 1622: *Cryptosporidium* in Water by Filtration/IMS/FA

1.0 Scope and Application

1.1 This method is for determination of the identity and concentration of *Cryptosporidium* (CAS Registry number 137259-50-8) in raw surface water and other waters by filtration, immunomagnetic separation (IMS), and immunofluorescence assay (FA) microscopy. *Cryptosporidium* may be confirmed using 4',6-diamidino-2-phenylindole (DAPI) staining and differential interference contrast (D.I.C.) microscopy.

1.2 This method is designed to meet the survey and monitoring requirements of the U.S. Environmental Protection Agency (EPA). It is based on laboratory testing of recommendations by a panel of experts convened by EPA. The panel was charged with recommending an improved protocol for recovery and detection of *Cryptosporidium* that could be tested and implemented with minimal additional research.

1.3 This method will not identify the species of *Cryptosporidium* or the host species of origin, nor can it determine the viability or infectivity of detected oocysts.

1.4 This method is for use only by persons experienced in the determination of *Cryptosporidium* by filtration, IMS, and FA. Experienced persons are defined in Section 22.0 at the end of this method as the principal analyst/supervisor, analyst, and technician. Laboratories unfamiliar with analyses of environmental samples by the techniques in this method should gain experience using water filtration techniques, IMS, fluorescent antibody staining with monoclonal antibodies, and microscopic examination of biological particulates using bright-field and D.I.C. microscopy.

1.5 Any modification of the method beyond those expressly permitted is subject to the application and approval of alternative test procedures under 40 *CFR* Part 141.27.

2.0 Summary of Method

2.1 A 10-L volume of water is collected in a carboy in the field and shipped to the laboratory. The sample is filtered in the laboratory and the oocysts and extraneous materials are retained on the filter.

2.2 Elution and separation

2.2.1 Materials on the filter are removed by elution with an aqueous buffered salt and detergent solution. The eluate is centrifuged to pellet the oocysts, and the supernatant fluid is aspirated.

2.2.2 The oocysts are magnetized by attachment of magnetic beads conjugated to an antibody. The magnetized oocysts are separated from the extraneous materials using a magnet, and the extraneous materials are discarded. The magnetic bead complex is then detached from the oocysts.

2.3 Enumeration

2.3.1 The oocysts are stained on well slides with fluorescently labeled monoclonal antibodies and 4',6-diamidino-2-phenylindole (DAPI) stain. The stained sample is examined using fluorescence and differential interference contrast (D.I.C.) microscopy.

2.3.2 Qualitative analysis is performed by scanning each slide well for objects that meet the size, shape, and fluorescence characteristics of *Cryptosporidium* oocysts. Potential oocysts are confirmed through DAPI staining characteristics and D.I.C. microscopy. An

oocyst is identified when size, shape color, and morphology agree with specified criteria and examples in a photographic library.

2.3.3 Quantitative analysis is performed by counting the total number of objects on the slide confirmed as oocysts.

2.4 Quality is assured through reproducible calibration and testing of the filtration, immunomagnetic separation (IMS), staining, and microscopy systems. Detailed information on these tests is provided in Section 9.0.

3.0 Definitions

3.1 *Cryptosporidium* is defined as a protozoan parasite potentially found in water and other media. The six species of *Cryptosporidium* and their potential hosts are *C. parvum* (mammals, including humans); *C. baileyi* and *C. meleagridis* (birds); *C. muris* (rodents); *C. serpentis* (reptiles); and *C. nasorum* (fish).

3.2 Definitions for other terms used in this method are given in the glossary (Section 22.0).

4.0 Contamination, Interferences, and Oocyst Degradation

4.1 Turbidity caused by inorganic and organic debris can interfere with the concentration, separation, and examination of the sample for *Cryptosporidium* oocysts. In addition to naturally-occurring debris, such as clays and algae, chemicals, such as iron and alum coagulants and polymers, may be added to water during the treatment process, and may result in additional interference.

4.2 Organisms and debris that autofluoresce or demonstrate non-specific fluorescence, such as algal and yeast cells, when examined by epifluorescent microscopy, may interfere with the detection of oocysts and contribute to false positives by immunofluorescent assay (FA).

4.3 Solvents, reagents, labware, and other sample-processing hardware may yield artifacts that may cause misinterpretation of microscopic examinations for oocysts. All materials used shall be demonstrated to be free from interferences under the conditions of analysis by running a method blank (negative control sample) initially and a minimum of once per week or after changes in source of reagent water. Specific selection of reagents and purification of solvents and other materials may be required.

4.4 Interferences co-extracted from samples will vary considerably from source to source, depending on the water being sampled. Experience suggests that high levels of algae, bacteria, and other protozoa can interfere in the identification of oocysts (Reference 20.1).

4.5 Freezing 10-L samples, filters, eluates, concentrates, or slides may interfere with the detection and/or identification of oocysts.

4.6 All equipment should be autoclaved after use and before washing. Clean equipment by scrubbing with warm detergent solution and exposing to hypochlorite solution (minimum of 5%) for at least 30 minutes at room temperature. Rinse the equipment with reagent water and place in an oocyst-free environment until dry. Disposable supplies should be used wherever possible.

5.0 Safety

5.1 The biohazard associated with, and the risk of infection from, oocysts is high in this method because live organisms are handled. This method does not purport to address all of the safety problems associated with its use. It is the responsibility of the laboratory to establish appropriate safety and health practices prior to use of this method. In particular, the analyst/technician must

know and observe the safety procedures required in a microbiology laboratory that handles pathogenic organisms while preparing, using, and disposing of sample concentrates, reagents and materials, and while operating sterilization equipment.

5.2 The toxicity or carcinogenicity of each compound or reagent used in this method has not been precisely determined; however, each chemical compound should be treated as a potential health hazard. Exposure to these compounds should be reduced to the lowest possible level. The laboratory is responsible for maintaining a current awareness file of Occupational Safety and Health Administration regulations regarding the safe handling of the chemicals specified in this method. A reference file of material safety data sheets should be made available to all personnel involved in these analyses. Additional information on laboratory safety can be found in References 20.2 through 20.5.

5.3 Samples may contain high concentrations of biohazards and toxic compounds, and must be handled with gloves and opened in a biological safety cabinet to prevent exposure. Reference materials and standards containing oocysts must also be handled with gloves and the analyst/technician must never place gloves in or near the face after exposure to solutions known or suspected to contain oocysts. Do not mouth-pipette.

5.4 Laboratory personnel must change gloves after handling filters and other contaminant-prone equipment and reagents. Gloves must be removed or changed before touching any other laboratory surfaces or equipment.

6.0 Equipment and Supplies

NOTE: Brand names, suppliers, and part numbers are for illustrative purposes only. No endorsement is implied. Equivalent performance may be achieved using apparatus and materials other than those specified here, but demonstration of equivalent performance that meets the requirements of this method is the responsibility of the laboratory.

6.1 Equipment for spiking samples in the laboratory

6.1.1 10-L carboy with bottom delivery port (½")—Cole-Palmer cat. no. 06080-42, or equivalent; calibrate to 10.0 L and mark level with waterproof marker

6.1.2 Stir bar—Fisher cat. no. 14-511-93, or equivalent

6.1.3 Stir plate—Fisher cat. no. 14-493-120S, or equivalent

6.1.4 Hemacytometer—Hauser Scientific, Horsham, PA, cat. no. 3200 or 1475, or equivalent

6.1.5 Hemacytometer coverslip—Hauser Scientific, cat. no. 5000 (for hemacytometer cat. no. 3200) or 1461 (for hemacytometer cat. no 1475), or equivalent

6.1.6 Lens paper without silicone—Fisher cat. no. 11-995, or equivalent

6.1.7 Polystyrene or polypropylene conical tubes with screw caps—15- and 50-mL

6.2 Equipment for laboratory filtration of samples

6.2.1 Capsule filter—Approximately 6-cm diameter × 21-cm long with approximately 1300 cm^2 polyethersulfone filter media and ½-in. inlet and outlet fittings, Pall Gelman Laboratory, Ann Arbor, MI, Envirochek™ Sampling Capsule, product 12110, or equivalent

6.2.2 Laboratory shaker with arms for agitation of sampling capsules

6.2.2.1 Laboratory shaker—Lab-Line model 3589, VWR Scientific cat. no. 57039-055, Fisher cat. no. 14260-11, or equivalent

6.2.2.2 Side arms for laboratory shaker—Lab-Line Model 3587-4, VWR Scientific cat. no. 57039-045, Fisher cat. no. 14260-13, or equivalent

6.3 Ancillary sampling equipment required for using capsule filter

6.3.1 Tubing—Glass, polytetrafluoroethylene (PTFE), high-density polyethylene (HDPE), or other tubing to which oocysts will not easily adhere—Tygon formula R-3603, or equivalent. If rigid tubing (glass, PTFE, HDPE) is used and the sampling system uses a peristaltic pump, a minimum length of compressible tubing may be used in the pump. Before use, the tubing must be thoroughly rinsed with detergent solution, followed by repeated rinsing with reagent water to minimize sample contamination.

6.3.2 Flow control valve—0.5 gpm (0.03 L/s), Bertram Controls, Plast-O-Matic cat. no. FC050B½-PV, or equivalent; or 0.4- to 4-Lpm flow meter with valve—Alamo Water Treatment, San Antonio, TX, cat. no. R5310, or equivalent

6.3.3 Centrifugal pump—Grainger, Springfield, VA, cat. no. 2P613, or equivalent

6.4 Immunomagnetic separation (IMS) apparatus

6.4.1 Sample mixer—Dynal Inc., Lake Success, NY, no. 947.01, or equivalent

6.4.2 Magnetic particle concentrator—For 10-mL test tubes, Dynal MPC-1®, no. 120.01, or equivalent

6.4.3 Magnetic particle concentrator—For microcentrifuge tubes, Dynal MPC-M®, no. 120.09, or equivalent

6.4.4 Flat-sided sample tubes—16 × 125 mm Leighton-type tubes with 60 ×10 mm flat-sided magnetic capture area, Dynal® L10, no. 740.03, or equivalent

6.5 Powder-free latex gloves—Fisher cat no. 113945B, or equivalent

6.6 Graduated cylinders, autoclavable—10-, 100-, and 1000-mL

6.7 Centrifuges

6.7.1 Centrifuge capable of accepting 15- to 250-mL conical centrifuge tubes and achieving 1100 × G —International Equipment Company, Needham Heights, MA, Centrifuge Size 2, Model K with swinging bucket, or equivalent

6.7.2 Centrifuge tubes—Conical, graduated, 1.5-, 50-, and 250-mL

6.8 Microscope

6.8.1 Epifluorescence/differential interference contrast (D.I.C.) with stage and ocular micrometers and 20X (N.A.=0.4) to 100X (N.A.=1.3) objectives—Zeiss™ Axioskop, Olympus™ BH, or equivalent

6.8.2 Excitation/band-pass filters for immunofluorescent assay (FA)—Zeiss™ 487909 or equivalent, including, 450- to 490-nm exciter filter, 510-nm dicroic beam-splitting mirror, and 515- to 520-nm barrier or suppression filter

6.8.3 Excitation/band-pass filters for DAPI—Filters cited below (Chroma Technology, Brattleboro, VT), or equivalent

Microscope model	Fluoro-chrome	Excitation filter (nm)	Dichroic beam-splitting mirror (nm)	Barrier or suppression filter (nm)	Chroma catalog number
Zeiss™ -Axioskop	DAPI (UV)	340-380	400	420	CZ902
Zeiss™ -IM35	DAPI (UV)	340-380	400	420	CZ702
Olympus™ BH	DAPI (UV)	340-380	400	420	11000
	Filter holder				91002
Olympus™ BX	DAPI (UV)	340-380	400	420	11000
	Filter holder				91008
Olympus™ IMT2	DAPI (UV)	340-380	400	420	11000
	Filter holder				91003

6.9 Ancillary equipment for microscopy

6.9.1 Well slides—Treated, 12-mm diameter, Meridian Diagnostics Inc., Cincinnati, OH, cat. no. R2206, or equivalent

6.9.2 Glass coverslips—22 × 50 mm

6.9.3 Fingernail polish—Clear or clear fixative, PGC Scientifics, Gaithersburg, MD, cat. no. 60-4890, or equivalent

6.9.4 Nonfluorescing immersion oil

6.9.5 Micropipette, adjustable: 0- to 10-μL with 0- to 10-μL tips
10- to 100-μL, with 10- to 200-μL tips
100- to 1000-μL with 100- to 1000-μL tips

6.9.6 Forceps—Splinter, fine tip

6.9.7 Forceps—Blunt-end

6.9.8 Desiccant—Drierite™ Absorbent, Fisher cat. no. 07-577-1A, or equivalent

6.10 Pipettes—glass or plastic

6.10.1 5-, 10-, and 25-mL

6.10.2 Pasteur, disposable

6.11 Balances

6.11.1 Analytical—Capable of weighing 0.1 mg

6.11.2 Top loading—Capable of weighing 10 mg

6.12 pH meter

6.13 Incubator—Fisher Scientific Isotemp™, or equivalent

6.14 Vortex mixer—Fisons Whirlmixer, or equivalent

6.15 Vacuum source—Capable of maintaining 25 in. Hg, equipped with shutoff valve and vacuum gauge

6.16 Miscellaneous labware and supplies

6.16.1 Test tubes and rack

6.16.2 Flasks—Suction, Erlenmeyer, and volumetric, various sizes

6.16.3 Beakers—Glass or plastic, 5-, 10-, 50-, 100-, 500-, 1000-, and 2000-mL

6.16.4 Lint-free tissues

6.17 10- to 15-L graduated container—Fisher cat. no. 02-961-50B, or equivalent; calibrate to 9.0, 9.5, 10.0, 10.5, and 11.0 L and mark levels with waterproof marker

6.18 Equipment for field sampling and shipping

6.18.1 10-L carboy—Cole Parmer cat. no. 06100-33, or equivalent

6.18.2 Shipping container—Cole Parmer cat. no. 06100-03, or equivalent

7.0 Reagents and Standards

7.1 Reagents for adjusting pH

7.1.1 Sodium hydroxide (NaOH)—ACS reagent grade, 6.0 N and 1.0 N in reagent water

7.1.2 Hydrochloric acid (HCl)—ACS reagent grade, 6.0 N, 1.0 N, and 0.1 N in reagent water

7.2 Solvents—Acetone, glycerol, ethanol, and methanol, ACS reagent grade

7.3 Reagent water—Water in which oocysts and interfering materials and substances, including magnetic minerals, are not detected by this method

7.4 Reagents for eluting capsule filters

7.4.1 Laureth-12—PPG Industries, Gurnee, IL, cat. no. 06194, or equivalent. Store Laureth-12 as a 10% solution in reagent water. Weigh 10 g of Laureth-12 and dissolve using a microwave or hot plate in 90 mL of reagent water. Dispense 10-mL aliquots into sterile vials and store at room temperature for up to 2 months, or in the freezer for up to a year.

7.4.2 1 M Tris, pH 7.4—Dissolve 121.1 g Tris (Fisher cat. no. BP152) in 700 mL of reagent water and adjust pH to 7.4 with 1 N HCl or NaOH. Dilute to a final 1000 mL with reagent water and adjust the final pH. Filter-sterilize through a 0.2-μm membrane into a sterile plastic container and store at room temperature.

7.4.3 0.5 M EDTA, 2 Na, pH 8.0—Dissolve 186.1 g ethylenediamine tetraacetic acid, disodium salt dihydrate (Fisher cat. no. S311) in 800 mL and adjust pH to 8.0 with 6.0 N HCl or NaOH. Dilute to a final volume of 1000 mL with reagent water and adjust to pH 8.0 with 1.0 N HCl or NaOH.

7.4.4 Antifoam A—Sigma Chemical Co. cat. no. A5758, or equivalent

7.4.5 Preparation of elution buffer solution—Add the contents of a pre-prepared Laureth-12 vial (Section 7.4.1) to a 1000-mL graduated cylinder. Rinse the vial several times to ensure the transfer of the detergent to the cylinder. Add 10 mL of Tris solution (Section 7.4.2), 2 mL of EDTA solution (Section 7.4.3), and 150 μL Antifoam A (Section 7.4.4). Dilute to 1000 mL with reagent water.

7.5 Reagents for immunomagnetic separation (IMS)—Dynabeads® anti-*Cryptosporidium* kit, Dynal cat no. 730.01, or equivalent

7.6 DABCO/glycerol mounting medium (2%)—Dissolve 2 g of DABCO (Sigma Chemical Co. cat no. D-2522, or equivalent) in 95 mL of warm glycerol/PBS (60% glycerol, 40% PBS [Section 7.8.3]). After the DABCO has dissolved completely, adjust the solution volume to 100 mL by adding an appropriate volume of glycerol/PBS solution.

7.7 Detection kit—Store the kit at 0°C to 8°C and return it promptly to this temperature after each use. Do not allow any of the reagents in this kit to freeze. The labeling reagents should be protected from exposure to light. Diluted, unused working reagents should be discarded after 48 hours. Discard the kit after the expiration date is reached.

7.7.1 Direct labeling kit for detection of oocysts—Crypt-a-Glo™, Waterborne, Inc., New Orleans, LA, cat. no. A400FL, or equivalent

7.7.2 4',6-diamidino-2-phenylindole (DAPI) stain

7.7.2.1 DAPI stock solution—Dissolve 2 mg/mL DAPI in absolute methanol. Prepare volume consistent with minimum use. Store at 0°C to 8°C in the dark. Discard unused solution after 2 weeks. Do not allow to freeze.

7.7.2.2 DAPI staining solution (1/5000 dilution in PBS [Section 7.8.3])—Add 10 μL of 2 mg/mL DAPI stock solution to 50 mL of PBS. Prepare daily. Store at 0°C to 8°C in the dark except when staining. Do not allow to freeze. The solution concentration may be increased up to 1 μg/mL if fading/diffusion of DAPI staining is encountered, but the staining solution must be tested first on expendable environmental samples to confirm that staining intensity is appropriate.

7.8 Oocyst suspension for spiking

7.8.1 Purified, live *Cryptosporidium* oocyst stock suspension—not heat-fixed, formalin-fixed, or treated in any way to reduce viability

7.8.2 Tween-20, 0.01%—Dissolve 1.0 mL of a 10% solution of Tween-20 in 1 L of reagent water.

7.8.3 Phosphate buffered saline (PBS), 150 mM—Add 1.07 g Na_2HPO_4, 0.39 g $NaH_2PO_4.2H_2O$, and 8.5 g NaCl to 800 mL reagent water. Dissolve and adjust to 1 L volume with reagent water. Adjust pH to 7.2 with NaOH or HCl. Prepare weekly.

7.8.4 Storage procedure—Store oocyst suspensions at 0°C to 8°C, until ready to use. Do not allow to freeze. Samples must be spiked: (1) within 24 hours of enumeration of the oocyst spiking suspension if the hemacytometer chamber technique is used (Section 11.3), or (2) within 24 hours of application of the spiking suspension to the 10 well slides if the well-slide enumeration technique is used (Section 11.4).

8.0 Sample Collection and Storage

8.1 Samples are collected in plastic 10-L carboys and shipped to the laboratory for filtration, elution, concentration, immunomagnetic separation (IMS), staining, and examination. Samples must be shipped to the laboratory the day they are collected and must arrive at the laboratory within 24 hours of sample collection. Store 10-L carboys at 0°C to 8°C between collection and shipment to the laboratory and upon receipt at the laboratory until ready for filtration. Do not allow to freeze.

NOTE: U.S. Department of Transportation (DOT) regulations (49 CFR 172) prohibit interstate shipment of more than 4 L of solution known to contain infectious materials. State regulations may contain similar regulations for intrastate commerce. This method requires a minimum sample volume of 10 L. Unless the sample is known or suspected to contain Cryptosporidium or other infectious agents (e.g., during an outbreak), samples should be shipped as noninfectious and should not be marked as infectious. If a sample is known or suspected to be infectious, and the sample must be shipped to a laboratory by a transportation means affected by DOT or state regulations, it is recommended that the sample be filtered in the field, and that the filter be shipped to the laboratory to avoid violating transport regulations.

8.2 Sample holding times: Laboratory filtration, elution, and concentration of a sample received in a carboy must be completed within 72 hours of sample collection. The concentrate must be stored at 0°C to 8°C if not proceding immediately to IMS. Do not allow to freeze.

8.3 Concentrate holding times: IMS and sample staining must be completed within 24 hours of completion of sample concentration. Stained slides must be stored at 0°C to 8°C in the dark. Do not allow to freeze.

8.4 Stained sample holding times: Although immunofluorescence assay (FA) and 4',6-diamidino-2-phenylindole (DAPI) and differential interference contrast (D.I.C.) microscopy examination and confirmation should be performed immediately after staining is complete, laboratories have up to 72 hours from completion of sample staining to complete the examination and confirmation of samples. However, if fading/diffusion of DAPI staining is noticed, the laboratory must reduce this holding time and/or adjust the concentration of the DAPI staining solution (Section 7.7.2.2) so that fading/diffusion does not occur.

8.5 Spiking suspension enumeration holding times: Initial and ongoing precision and recovery (IPR and OPR) samples and matrix spike (MS) samples must be spiked: (1) within 24 hours of enumeration of the oocyst spiking suspension if the hemacytometer chamber technique is used (Section 11.3), or (2) within 24 hours of application of the spiking suspension to the 10 well slides if the well-slide enumeration technique is used (Section 11.4).

9.0 Quality Control

9.1 Each laboratory that uses this method is required to operate a formal quality assurance (QA) program (Reference 20.6). The minimum requirements of this program consist of an initial demonstration of laboratory capability through performance of the initial precision and recovery test (Section 9.4), analysis of spiked samples to evaluate and document data quality, and analysis of standards and blanks as tests of continued performance. Laboratory performance is compared to established performance criteria to determine if the results of analyses meet the performance characteristics of the method.

9.1.1 A test of the microscope used for detection of oocysts is performed prior to examination of slides. This test is described in Section 10.0.

9.1.2 In recognition of advances that are occurring in analytical technology, the laboratory is permitted to modify certain method procedures to improve recovery or lower the costs of measurements, provided that all quality control (QC) tests cited in Section 9.1.2.1 are performed and all QC acceptance criteria (Table 1 in Section 21.0) are met. Method procedures that can be modified include front-end techniques, such as filtration or immunomagnetic separation (IMS). The laboratory is not permitted to use an alternate determinative technique (such as polymerase chain reaction) to replace immunofluorescent assay in this method. However the laboratory is permitted to modify the immunofluorescent assay procedure, provided that all QC tests cited in Section 9.1.2.1 are performed and all QC acceptance criteria are met.

9.1.2.1 Each time a modification is made to this method, the laboratory is required to perform the IPR test (Section 9.4) and the matrix spike/matrix spike duplicate (MS/MSD) test (Section 9.5) to demonstrate that the modification produces results equivalent or superior to results produced by this method.

9.1.2.2 The laboratory is required to maintain records of modifications made to this method. These records include the following, at a minimum:

9.1.2.2.1 The names, titles, addresses, and telephone numbers of the analyst(s) who performed the analyses and modification, and of the quality control officer who witnessed and will verify the analyses and modification.

9.1.2.2.2 A listing of the analyte(s) measured (*Cryptosporidium*).

9.1.2.2.3 A narrative stating reason(s) for the modification.

9.1.2.2.4 Results from all QC tests comparing the modified method to this method, including:

(a) Microscope calibration (Section 10.0)
(b) Calibration verification (Section 10.0)
(c) IPR (Section 9.4)
(d) MS/MSD (Section 9.5)
(e) Analysis of method blanks (Section 9.6)

9.1.2.2.5 Data that will allow an independent reviewer to validate each determination by tracing the following processing and analysis steps leading to the final result:

(a) Sample numbers and other identifiers
(b) Source of spiking suspension, as well as lot number and date received (Section 7.8)
(c) Spike enumeration date and time
(d) All spiking suspension enumeration counts and calculations (Section 11.0)
(e) Sample spiking dates and times
(f) Volume filtered (Section 12.2.5.2)
(g) Filtration and concentration dates and times
(h) Initial pellet volume and resuspended pellet volume(s) (Section 13.2)
(i) Staining completion dates and times
(j) Staining control results (Section 15.2.1)
(k) All required examination and confirmation information (Section 15.2)
(l) Examination and confirmation dates and times
(m) Analysis sequence/run chronology
(n) Lot numbers of elution, IMS, and staining reagents
(o) Copies of bench sheets, logbooks, and other recordings of raw data
(p) Data system outputs, and other data to link the raw data to the results reported

9.1.3 The laboratory shall spike a separate sample aliquot from the same source to monitor method performance. This MS test is described in Section 9.5.1.

9.1.4 Analysis of method blanks is required to demonstrate freedom from contamination. The procedures and criteria for analysis of a method blank are described in Section 9.6.

9.1.5 The laboratory shall, on an ongoing basis, demonstrate through analysis of the ongoing precision and recovery (OPR) sample that the analysis system is in control. These procedures are described in Section 9.7.

9.1.6 The laboratory shall maintain records to define the quality of data that are generated. Development of accuracy statements is described in Sections 9.5.1.4 and 9.7.3.

9.1.7 The laboratory shall analyze one method blank (Section 9.6) and one OPR sample (Section 9.7) each week during which samples are analyzed if 20 or fewer field samples are analyzed during this period. The laboratory shall analyze one laboratory blank and one OPR sample for every 20 samples if more than 20 samples are analyzed in a week.

9.1.8 The laboratory shall analyze one MS sample (Section 9.5.1) when samples are first received from a utility for which the laboratory has never before analyzed samples. The MS analysis is performed on an additional (second) sample sent from the utility. If the laboratory routinely analyzes samples from 1 or more utilities, 1 MS analysis must be

performed per 20 field samples. For example, when a laboratory receives the first sample from a given site, the laboratory must obtain a second aliquot of this sample to be used for the MS. When the laboratory receives the 21st sample from this site, a separate aliquot of this 21st sample must be collected and spiked.

9.2 Micropipette calibration

9.2.1 Micropipettes must be sent to the manufacturer for calibration annually. Alternately, a qualified independent technician specializing in micropipette calibration can be used. Documentation on the precision of the recalibrated micropipette must be obtained from the manufacturer or technician.

9.2.2 Internal and external calibration records must be kept on file in the laboratory's QA logbook.

9.2.3 If a micropipette calibration problem is suspected, the laboratory shall tare an empty weighing boat on the analytical balance and pipette the following volumes of reagent water into the weigh boat using the pipette in question: 100% of the maximum dispensing capacity of the micropipette, 50% of the capacity, and 10% of the capacity. Record the weight of the water (assume that 1.00 mL of reagent water weighs 1.00 g). If the weight of the reagent water is within 1% of the desired weight (mL) then the pipette remains acceptable for use.

9.2.4 If the weight of the reagent water is outside the acceptable limits, consult the manufacturer's instruction manual troubleshooting section and repeat steps described in Section 9.2.3. If problems with the pipette persist, the laboratory must send the pipette to the manufacturer for recalibration.

9.3 Microscope adjustment and certification: Adjust the microscope as specified in Section 10.0. All of the requirements in Section 10.0 must be met prior to analysis of IPRs, blanks, OPRs, field samples, and MS/MSDs.

9.4 Initial precision and recovery (IPR)—To establish the ability to demonstrate control over the analytical system and to generate acceptable precision and recovery, the laboratory shall perform the following operations:

9.4.1 Using the spiking procedure in Section 11.5 and enumerated spiking suspension aliquots containing 100 to 500 oocysts (Section 11.3 or 11.4), the laboratory must filter, elute, concentrate, separate (purify), stain, and examine four 10-L reagent water samples. If more than one process will be used for filtration and/or separation of samples, a separate set of IPR samples must be prepared for each process.

NOTE: IPR tests must be accompanied by analysis of a method blank (Section 9.6).

9.4.2 Using results of the four analyses, compute the average percent recovery (X) and the relative standard deviation of the recovery (s_r) for *Cryptosporidium*.

9.4.3 Compare s_r and X with the corresponding limits for initial precision and recovery in Table 1 in Section 21.0. If s_r and X meet the acceptance criteria, system performance is acceptable and analysis of blanks and samples may begin. If s_r or X falls outside the range for recovery, system performance is unacceptable. In this event, correct the problem and repeat the test (Section 9.4.1).

9.5 Matrix spike (MS) and matrix spike duplicate (MSD):

9.5.1 Matrix spike—The laboratory shall spike and analyze a separate field sample aliquot to determine the effect of the matrix on the method's oocyst recovery. The MS shall be analyzed according to the frequency in Section 9.1.8.

9.5.1.1 Analyze an unspiked field sample according to the procedures in Sections 12.0 to 15.0. Using the spiking procedure in Section 11.5 and an

appropriate volume of enumerated oocyst spiking suspension (Section 11.3 or 11.4), spike a second field sample aliquot to produce five times the number of oocysts detected in the unspiked sample or the number used in the IPR or OPR tests (Sections 9.4 and 9.7), whichever is greater.

9.5.1.2 Compute the percent recovery (R) of protozoa using the following equation.

$$R = 100 \times \frac{N_{sp} - N_s}{T}$$

where

R is the percent recovery
N_{sp} is the number of oocysts detected in the spiked sample
N_s is the number of oocysts detected in the unspiked sample
T is the true value of the oocysts spiked

9.5.1.3 Compare the protozoa recovery with the corresponding limits in Table 1 in Section 21.0. If the recovery for *Cryptosporidium* oocysts falls outside its limit, method performance is unacceptable for that sample. If the results for the method blank (Section 9.6) and for the OPR sample (Section 9.7) associated with this batch of samples are within their respective control limits, a matrix interference may be causing the poor recovery. See Section 16.0 for instructions for dealing with matrix interferences; however the matrix may not be diluted for MS tests. If the results for the blank and OPR are not within their control limits, the laboratory is not in control. The problem must be identified and corrected and a fresh sample should be collected and reanalyzed.

9.5.1.4 As part of the QA program for the laboratory, method precision for samples should be assessed and records maintained. After the analysis of five samples for which the spike recovery for *Cryptosporidium* passes the tests in Section 9.5.1.3, the laboratory should compute the average percent recovery (P) and the standard deviation of the percent recovery (s_r). Express the precision assessment as a percent recovery interval from P – 2 s_r to P + 2 s_r for each matrix. For example, if P = 80% and s_r = 30%, the accuracy interval is expressed as 20% to 140%. The precision assessment should be updated on a regular basis (e.g., after each 5 to 10 new accuracy measurements).

9.5.2 Matrix spike duplicate—MSD analysis is required to demonstrate that a modified version of this method produces results equal or superior to results produced by the method as written (Section 9.1.2). At the same time the laboratory spikes and analyzes the second field sample aliquot in Section 9.5.1.1, the laboratory shall spike and analyze an third, identical field sample aliquot.

9.5.2.1 Calculate the percent recovery (R) for *Cryptosporidium* using the equation in Section 9.5.1.2. Calculate the mean of the MS and MSD recoveries (X_{mean}) (= [MS+MSD]/2).

9.5.2.2 Calculate the relative percent difference (RPD) of the recoveries using the following equation:

$$RPD = 100 \frac{|R_{MS} - R_{MSD}|}{X_{mean}}$$

where

RPD is the relative percent difference
R_{MS} is the number of oocysts detected in the MS

R_{MSD} is the number of oocysts detected in the MSD
X_{mean} is the mean of the recoveries for the MS and MSD

9.5.2.3 Compare the mean MS/MSD recovery and RPD with the corresponding limits in Table 1 in Section 21.0.

9.6 Method blank (negative control sample): Reagent water blanks are analyzed to demonstrate freedom from contamination. Analyze the blank immediately prior to analysis of the IPR test (Section 9.4) and OPR test (Section 9.7) and prior to analysis of samples for the week to demonstrate freedom from contamination.

9.6.1 Filter, elute, concentrate, separate (purify), stain, and examine at least one reagent water blank per week (Section 9.1.7) according to the procedures in Sections 12.0 to 15.0. If more than 20 samples are analyzed in a week, process and analyze one reagent water blank for every 20 samples.

9.6.2 If *Cryptosporidium* oocysts or any potentially interfering organism or material is found in the blank, analysis of additional samples is halted until the source of contamination is eliminated and a blank shows no evidence of contamination. Any sample in a batch associated with a contaminated blank that shows the presence of one or more oocysts is assumed to be contaminated and must be recollected. Any method blank in which oocysts are not detected is assumed to be uncontaminated and may be reported.

9.7 Ongoing precision and recovery ([OPR]; positive control sample; laboratory control sample): Using the spiking procedure in Section 11.5 and enumerated spiking suspension containing 100 to 500 oocysts (Section 11.3 or 11.4), filter, elute, concentrate, separate (purify), stain, and examine at least one spiked reagent water sample per week to verify all performance criteria. The laboratory must analyze one OPR sample for every 20 samples if more than 20 samples are analyzed in a week. Adjustment and/or recalibration of the analytical system shall be performed until all performance criteria are met. Only after all performance criteria are met may samples be analyzed.

9.7.1 Examine the slide from the OPR prior to analysis of samples from the same batch.

9.7.1.1 More than 50% of the oocysts must appear undamaged and morphologically intact; otherwise, the analytical process is damaging the oocysts. Determine the step or reagent that is causing damage to the oocysts. Correct the problem and repeat the OPR test.

9.7.1.2 Identify and enumerate each organism using epifluorescence microscopy. Each organism must meet the identification criteria in Section 15.2.

9.7.2 Compute the percent recovery of the total number of oocysts using the following equation:

$$P = 100 \times \frac{N}{T}$$

where
N = the number of oocysts detected
T = the number of oocysts spiked

9.7.2.1 Compare the recovery with the limits for ongoing precision and recovery in Table 1 in Section 21.0. If the recovery meets the acceptance criteria, system performance is acceptable and analysis of blanks and samples may proceed. If, however, the recovery falls outside of the range given, system performance is unacceptable. In this event, there may be a problem with the microscope or with the filtration or separation systems. Reanalyze the OPR sample and recollect and reanalyze samples. All

samples must be associated with an OPR that passes the criteria in Section 21.0.

9.7.2.2 Microscope system: To determine if the failure of the OPR test (Section 9.7.2.1) is due to changes in the microscope, examine a slide containing a known number of freshly prepared oocysts, check Köhler illumination, and check the fluorescence of the fluorescein-labeled monoclonal antibody (Mab) and 4',6-diamidino-2-phenylindole (DAPI).

9.7.2.3 Filtration/elution/concentration system: If the failure of the OPR test (Section 9.7.2.1) is attributable to the filtration/elution/concentration system, these systems may not be in control. Check filtration/elution/ concentration system performance using spiked reagent water, eluting the filter, and analyzing the concentrated sample without separation (purification) using FA.

9.7.2.4 Separation (purification) system: If the failure of the OPR test (Section 9.7.2.1) is attributable to the separation system, this system may not be in control. Check separation system performance by processing a spiked 10-mL volume of reagent water through IMS and analyzing the purified sample using FA (Sections 13.3 through 15.0).

9.7.3 The laboratory should add results that pass the specifications in Section 9.7.2.1 to initial and previous ongoing data and update the QC chart to form a graphic representation of continued laboratory performance. The laboratory should develop a statement of laboratory accuracy (reagent water, raw surface water) by calculating the average percent recovery (R) and the standard deviation of percent recovery (s_r). Express the accuracy as a recovery interval from R – 2 s_r to R + 2 s_r. For example, if R = 95% and s_r = 25%, the accuracy is 45% to 145%.

9.8 The laboratory should periodically analyze an external QC sample, such as a performance evaluation or standard reference material, when available. The laboratory also should periodically participate in interlaboratory comparison studies using the method.

9.9 The specifications contained in this method can be met if the analytical system is under control. The standards used for initial (Section 9.4) and ongoing (Section 9.7) precision and recovery should be identical, so that the most precise results will be obtained. The microscope in particular will provide the most reproducible results if dedicated to the settings and conditions required for the determination of *Cryptosporidium* by this method.

9.10 Depending on specific program requirements, field replicates may be collected to determine the precision of the sampling technique, and duplicate spiked samples may be required to determine the precision of the analysis.

10.0 Microscope Calibration and Analyst Verification

10.1 In a room capable of being darkened to near-complete darkness, assemble the microscope, all filters, and attachments. The microscope should be placed on a solid surface free from vibration. Adequate workspace should be provided on either side of the microscope for taking notes and placement of slides and ancillary materials.

10.2 Using the manuals provided with the microscope, the principal analyst/supervisor and all analysts must familiarize themselves with operation of the microscope.

10.3 Microscope adjustment and calibration (adapted from Reference 20.6)

10.3.1 Preparations for adjustment

10.3.1.1 The microscopy portion of this procedure depends upon proper alignment and adjustment of very sophisticated optics. Without proper

alignment and adjustment, the microscope will not function at maximal efficiency, and reliable identification and enumeration of oocysts will not be possible. Consequently, it is imperative that all portions of the microscope from the light sources to the oculars are properly adjusted.

10.3.1.2 While microscopes from various vendors are configured somewhat differently, they all operate on the same general physical principles. Therefore, slight deviations or adjustments may be required to make the procedures below work for a particular instrument.

10.3.1.3 The sections below assume that the mercury bulb has not exceeded time limits of operation, that the lamp socket is connected to the lamp house, and that the condenser is adjusted to produce Köhler illumination.

10.3.1.4 Persons with astigmatism should always wear contact lenses or glasses when using the microscope.

CAUTION: In the procedures below, do not touch the quartz portion of the mercury bulb with your bare fingers. Finger oils can cause rapid degradation of the quartz and premature failure of the bulb.

WARNING: Never look at the ultraviolet (UV) light from the mercury lamp, lamp house, or the UV image without a barrier filter in place. UV radiation can cause serious eye damage.

10.3.2 Epifluorescent mercury bulb adjustment: The purpose of this procedure is to ensure even field illumination. This procedure must be followed when the microscope is first used, when replacing bulbs, and if problems such as diminished fluorescence or uneven field illumination are experienced.

10.3.2.1 Remove the diffuser lens between the lamp and microscope or swing it out of the transmitted light path.

10.3.2.2 Using a prepared microscope slide, adjust the focus so the image in the oculars is sharply defined.

10.3.2.3 Replace the slide with a business card or a piece of lens paper.

10.3.2.4 Close the field diaphragm (iris diaphragm in the microscope base) so only a small point of light is visible on the card. This dot of light indicates the location of the center of the field of view.

10.3.2.5 Mount the mercury lamp house on the microscope without the UV diffuser lens in place and turn on the mercury bulb.

10.3.2.6 Remove the objective in the light path from the nosepiece. A primary (brighter) and secondary image (dimmer) of the mercury bulb arc should appear on the card after focusing the image with the appropriate adjustment.

10.3.2.7 Using the lamp house adjustments, adjust the primary and secondary mercury bulb images so they are side by side (parallel to each other) with the transmitted light dot in between them.

10.3.2.8 Reattach the objective to the nosepiece.

10.3.2.9 Insert the diffuser lens into the light path between the mercury lamp house and the microscope.

10.3.2.10 Turn off the transmitted light and replace the card with a slide of fluorescent material. Check the field for even fluorescent illumination.

Adjustment of the diffuser lens probably will be required. Additional slight adjustments as in Section 10.3.2.7 above may be required.

10.3.2.11 Maintain a log of the number of hours the UV bulb has been used. Never use the bulb for longer than it has been rated. Fifty-watt bulbs should not be used longer than 100 hours; 100-watt bulbs should not be used longer than 200 hours.

10.3.3 Transmitted bulb adjustment: The purpose of this procedure is to center the filament and ensure even field illumination. This procedure must be followed when the bulb is changed.

10.3.3.1 Remove the diffuser lens between the lamp and microscope or swing it out of the transmitted light path.

10.3.3.2 Using a prepared microscope slide and a 40X (or similar) objective, adjust the focus so the image in the oculars is sharply defined.

10.3.3.3 Without the ocular or Bertrand optics in place, view the pupil and filament image at the bottom of the tube.

10.3.3.4 Focus the lamp filament image with the appropriate adjustment on the lamp house.

10.3.3.5 Similarly, center the lamp filament image within the pupil with the appropriate adjustment(s) on the lamp house.

10.3.3.6 Insert the diffuser lens into the light path between the transmitted lamp house and the microscope.

10.3.4 Adjustment of the interpupillary distance and oculars for each eye: These adjustments are necessary so that eye strain is reduced to a minimum, and must be made for each individual using the microscope. Section 10.3.4.2 assumes use of a microscope with both oculars adjustable; Section 10.3.4.3 assumes use of a microscope with a single adjustable ocular. The procedure must be followed each time an analyst uses the microscope.

10.3.4.1 Interpupillary distance

10.3.4.1.1 Place a prepared slide on the microscope stage, turn on the transmitted light, and focus the specimen image using the coarse and fine adjustment knobs.

10.3.4.1.2 Using both hands, move the oculars closer together or farther apart until a single circle of light is observed while looking through the oculars with both eyes. Note interpupillary distance.

10.3.4.2 Ocular adjustment for microscopes capable of viewing a photographic frame through the viewing binoculars: This procedure assumes both oculars are adjustable.

10.3.4.2.1 Place a card between the right ocular and eye keeping both eyes open. Adjust the correction (focusing) collar on the left ocular by focusing the left ocular until it reads the same as the interpupillary distance. Bring an image located in the center of the field of view into as sharp a focus as possible.

10.3.4.2.2 Transfer the card to between the left eye and ocular. Again keeping both eyes open, bring the same image into as sharp a focus for the right eye as possible by

adjusting the ocular correction (focusing) collar at the top of the right ocular.

10.3.4.3 Ocular adjustment for microscopes without binocular capability: This procedure assumes a single focusing ocular. The following procedure assumes that only the right ocular is capable of adjustment.

10.3.4.3.1 Place a card between the right ocular and eye keeping both eyes open. Using the fine adjustment, focus the image for the left eye to its sharpest point.

10.3.4.3.2 Transfer the card to between the left eye and ocular. Keeping both eyes open, bring the image for the right eye into sharp focus by adjusting the ocular collar at the top of the ocular without touching the coarse or fine adjustment.

10.3.5 Calibration of an ocular micrometer: This section assumes that a reticle has been installed in one of the oculars by a microscopy specialist and that a stage micrometer is available for calibrating the ocular micrometer (reticle). Once installed, the ocular reticle should be left in place. The more an ocular is manipulated the greater the probability is for it to become contaminated with dust particles. This calibration should be done for each objective in use on the microscope. If there is a top lens on the microscope, the calibration procedure must be done for the respective objective at each top lens setting. The procedure must be followed when the microscope is first used and each time the objective is changed.

10.3.5.1 Place the stage micrometer on the microscope stage, turn on the transmitted light, and focus the micrometer image using the coarse and fine adjustment knobs for the objective to be calibrated. Continue adjusting the focus on the stage micrometer so you can distinguish between the large (0.1 mm) and the small (0.01 mm) divisions.

10.3.5.2 Adjust the stage and ocular with the micrometer so the 0 line on the ocular micrometer is exactly superimposed on the 0 line on the stage micrometer.

10.3.5.3 Without changing the stage adjustment, find a point as distant as possible from the two 0 lines where two other lines are exactly superimposed.

10.3.5.4 Determine the number of ocular micrometer spaces as well as the number of millimeters on the stage micrometer between the two points of superimposition. For example: Suppose 48 ocular micrometer spaces equal 0.6 mm.

10.3.5.5 Calculate the number of mm/ocular micrometer space. For example:

$$\frac{0.6\ mm}{48\ ocular\ micrometer\ spaces} = \frac{0.0125\ mm}{ocular\ micrometer\ space}$$

10.3.5.6 Because most measurements of microorganisms are given in μm rather than mm, the value calculated above must be converted to μm by multiplying it by 1000 μm/mm. For example:

$$\frac{0.0125\ mm}{ocular\ micrometer\ space} \times \frac{1{,}000\ \mu m}{mm} = \frac{12.5\ \mu m}{ocular\ micrometer\ space}$$

10.3.5.7 Follow the procedure below for each objective. Record the information as shown in the example below and keep the information available at the microscope.

Item no.	Objective power	Description	No. of ocular micrometer spaces	No. of stage micrometer mm[1]	μm/ocular micrometer space[2]
1	10X	N.A.[3]=			
2	20X	N.A.=			
3	40X	N.A.=			
4	100X	N.A.=			

[1]100 μm/mm

[2](Stage micrometer length in mm × (1000 μm/mm)) ÷ no. ocular micrometer spaces

[3]N.A. refers to numerical aperture. The numerical aperature value is engraved on the barrel of the objective.

10.3.6 Köhler illumination: This section assumes that Köhler illumination will be established for only the 100X oil D.I.C. objective that will be used to identify internal morphological characteristics in *Cryptosporidium* oocysts. If more than one objective is to be used for D.I.C., then each time the objective is changed, Köhler illumination must be reestablished for the new objective lens. Previous sections have adjusted oculars and light sources. This section aligns and focuses the light going through the condenser underneath the stage at the specimen to be observed. If Köhler illumination is not properly established, then D.I.C. will not work to its maximal potential. These steps need to become second nature and must be practiced regularly until they are a matter of reflex rather than a chore. The procedure must be followed each time an analyst uses the microscope and each time the objective is changed.

10.3.6.1 Place a prepared slide on the microscope stage, place oil on the slide, move the 100X oil objective into place, turn on the transmitted light, and focus the specimen image using the coarse and fine adjustment knobs.

10.3.6.2 At this point both the radiant field diaphragm in the microscope base and the aperture diaphragm in the condenser should be wide open. Now close down the radiant field diaphragm in the microscope base until the lighted field is reduced to a small opening.

10.3.6.3 Using the condenser centering screws on the front right and left of the condenser, move the small lighted portion of the field to the center of the visual field.

10.3.6.4 Now look to see whether the leaves of the iris field diaphragm are sharply defined (focused) or not. If they are not sharply defined, then they can be focused distinctly by changing the height of the condenser up and down with the condenser focusing knob while you are looking through the binoculars. Once you have accomplished the precise focusing of the radiant field diaphragm leaves, open the radiant field diaphragm until the leaves just disappear from view.

10.3.6.5 The aperture diaphragm of the condenser is now adjusted to make it compatible with the total numerical aperture of the optical system. This is done by removing an ocular, looking into the tube at the rear focal plane of the objective, and stopping down the aperture diaphragm iris leaves until they are visible just inside the rear plane of the objective.

10.3.6.6 After completing the adjustment of the aperture diaphragm in the condenser, return the ocular to its tube and proceed with the adjustments required to establish D.I.C.

10.4 Protozoa libraries: Each laboratory is encouraged to develop libraries of photographs and drawings for identification of protozoa.

10.4.1 Take color photographs of *Cryptosporidium* oocysts by FA and 4',6-diamidino-2-phenylindole (DAPI) that the principal analyst/supervisor (Section 22.2) determines are accurate (Section 15.2).

10.4.2 Similarly, take color photographs of interfering organisms and materials by FA and DAPI that the principal analyst/supervisor believes are not *Cryptosporidium* oocysts. Quantify the size, shape, microscope settings, and other characteristics that can be used to differentiate oocysts from interfering debris and that will result in positive identification of DAPI + or - organisms.

10.5 Verification of performance: Until standard reference materials, such as National Institute of Standards and Technology standard reference materials, are available that contain a reliable number of DAPI + or - oocysts, this method shall rely upon the ability of the principal analyst/supervisor for identification and enumeration of oocysts.

10.5.1 At least monthly when microscopic examinations are being performed, the principal analyst/supervisor shall prepare a slide containing 40 to 100 oocysts. More than 50% of the oocysts must be DAPI +. The principal analyst/supervisor shall determine the numbers of total oocysts by FA and number of oocysts that are DAPI + or -, using the procedures in this method, and these numbers shall be known only to the principal analyst/supervisor.

10.5.2 Each analyst shall determine the total number of oocysts and the number that are DAPI + or -, using the slide provided by the principal analyst/supervisor (Section 10.5.1).

10.5.3 The total number and the number of DAPI + or - oocysts determined by each analyst (Section 10.5.2.) must be within ±10% of the number determined by the principal analyst/supervisor. If the number is not within this range, the principal analyst/ supervisor and the analyst shall resolve how to identify and enumerate DAPI + or - oocysts, and the principal analyst/supervisor shall prepare a new slide and the performance verification (Sections 10.5.1 to 10.5.2) shall be repeated.

NOTE: If the laboratory has only a principal analyst/supervisor, the principal analyst/supervisor shall perform the identification and enumeration of total and DAPI + and - oocysts on a monthly basis, at a minimum, and the reputation of the laboratory shall rest with the principal analyst/supervisor.

10.5.4 Document the date, name of principal analyst/supervisor, name(s) of analyst(s), number of total, DAPI + or - oocysts placed on the slide, number determined by the principal analyst/supervisor, number determined by the analyst(s), whether the test was passed/failed for each analyst, and the number and results of attempts prior to passage.

10.5.5 Only after an analyst has passed the criteria in Section 10.5.3, may oocysts in QC samples and field samples be identified and enumerated.

11.0 Oocyst Suspension Enumeration and Spiking

11.1 Two sets of enumerations are required before purified oocyst stock suspensions received from suppliers can be used to spike samples in the laboratory. First, the oocyst stock suspension must be diluted and enumerated to yield an oocyst suspension at the appropriate concentration for spiking (oocyst spiking suspension). Then, 10 aliquots of oocyst spiking suspension must be

enumerated to calculate a mean spike dose. Oocyst spiking suspensions can be enumerated using either hemacytometer chamber counting or well-slide counting. The procedure for diluting and enumerating purified oocyst stock suspensions is provided in Section 11.2. The two procedures for enumerating oocyst spiking suspensions are provided in Sections 11.3 and 11.4. The procedure for spiking 10-L carboys in the laboratory is provided in Section 11.5.

11.2 Enumerating and diluting the oocyst stock suspension

11.2.1 Purified, concentrated *Cryptosporidium* oocyst stock suspension (Section 7.8.1) must be diluted and enumerated before the diluted suspension is used to spike samples in the laboratory. Stock suspension should be diluted with reagent water/Tween-20, 0.01% (Section 7.8.2), to a concentration of 20 to 50 oocysts per large hemacytometer square before proceeding to Section 11.2.2.

11.2.2 Apply a clean hemacytometer coverslip to the hemacytometer and load the hemacytometer chamber with 10 μL of vortexed oocyst suspension per chamber. If this operation has been properly executed, the liquid should amply fill the entire chamber without bubbles or overflowing into the surrounding moats. Repeat this step with a clean, dry hemacytometer and coverslip if loading has been incorrectly performed. See Section 11.2.13, below, for the hemacytometer cleaning procedure.

11.2.3 Place the hemacytometer on the microscope stage and allow the oocysts to settle for 2 minutes. Do not attempt to adjust the coverslip, apply clips, or in any way disturb the chamber after it has been filled.

11.2.4 Use a magnification of 400X to 500X.

11.2.5 Move the chamber so the ruled area is centered underneath it.

11.2.6 Move the objective close to the coverslip while watching it from the side of the microscope, rather than through the microscope.

11.2.7 Focus up from the coverslip until the hemacytometer ruling appears.

11.2.8 At each of the four corners of the chamber is a 1-square-mm area divided into 16 squares in which oocysts are to be counted (Figure 1). Beginning with the top row of four squares, count with a hand-tally counter in the directions indicated in Figure 2. Avoid counting oocysts twice by counting only those touching the top and left boundary lines. Count each square millimeter in this fashion.

11.2.9 Use the following formula to determine the number of oocysts per mL of suspension:

$$\frac{\textit{number of oocysts counted}}{\textit{number of } mm^2 \textit{ counted}} \times \frac{10}{1\,mm} \times \frac{\textit{dilution factor}}{1} \times \frac{1000\ mm^3}{1\,mL} = \textit{number of oocysts/mL}$$

11.2.10 Record the result on a hemacytometer data sheet.

11.2.11 A total of six different hemacytometer chambers must be loaded, counted, and averaged for each oocyst suspension to achieve optimal counting accuracy.

11.2.12 Based on the hemacytometer counts, the stock suspension should be diluted to a final concentration of between 8000 and 12,000 oocysts per mL (80 to 120 oocysts per 10 μL); however, ranges as great as 5000 to 15,000 oocysts per mL (50 to 150 oocysts per 10 μL) can be used.

NOTE: If the diluted stock suspension (the spiking suspension) will be enumerated using hemacytometer chamber counts (Section 11.3), then the stock suspension should be diluted with reagent water/Tween-20, 0.01%. If the spiking suspension will be enumerated using well-slide counts (Section 11.4), or if both hemacytometer chamber counts and well-slide counts will be used to enumerate the oocyst spiking suspension, then the stock suspension should be diluted using reagent water only.

To calculate the volume (in μL) of stock oocyst suspension required per mL of reagent water (or reagent water/Tween-20, 0.01%), use the following formula:

$$volume\ of\ stock\ suspension\ (mL)\ required = \frac{required\ number\ of\ oocysts \times 1000\ mL}{number\ of\ oocysts\ /\ mL\ of\ stock\ suspension}$$

If the volume is less than 10 μL, an additional dilution of the stock suspension is recommended before proceeding.

To calculate the dilution factor needed to achieve the required number of oocysts per 10 μL, use the following formula:

$$total\ volume\ (mL) = \frac{number\ of\ oocysts\ required \times 10\ mL}{predicted\ number\ of\ oocysts\ per\ 10\ mL\ (80\ to\ 120)}$$

To calculate the volume of reagent water (or reagent water/Tween-20, 0.01%) needed, use the following formula:

$$reagent\ water\ volume\ (mL) = total\ volume\ (mL) - stock\ oocyst\ suspension\ volume\ required\ (mL)$$

11.2.13 After each use, the hemacytometer and coverslip must be cleaned immediately to prevent the oocysts and debris from drying on it. Since this apparatus is precisely machined, abrasives cannot be used to clean it, as they will disturb the flooding and volume relationships.

11.2.13.1 Rinse the hemacytometer and cover glass first with tap water, then 70% ethanol, and finally with acetone.

11.2.13.2 Dry and polish the hemacytometer chamber and cover glass with lens paper. Store it in a secure place.

11.2.14 Several factors are known to introduce errors into hemacytometer counts, including:

- Inadequate mixing of suspension before flooding the chamber
- Irregular filling of the chamber, trapped air bubbles, dust, or oil on the chamber or coverslip
- Total number of oocysts counted is too low to provide statistical confidence in the result
- Error in recording tally
- Calculation error; failure to consider dilution factor, or area counted
- Inadequate cleaning and removal of oocysts from the previous count
- Allowing filled chamber to sit too long, so that the chamber suspension dries and concentrates.

11.3 Enumerating the oocyst spiking suspension using a hemacytometer chamber

11.3.1 Vortex the tube containing oocyst spiking suspension (diluted stock oocyst suspension; Section 11.2) for a minimum of 2 minutes. Gently invert the tube three times.

11.3.2 To an appropriate-size beaker containing a stir bar, add enough oocyst spiking suspension to perform all spike testing and the enumeration as described. The liquid volume and beaker relationship should be such that a spinning stir bar does not splash the sides of the beaker, the stir bar has unimpeded rotation, and there is enough room to draw sample from the beaker with a 10-μL micropipette without touching the stir bar. Cover the beaker with a watch glass or petri dish to prevent evaporation between sample withdrawals.

11.3.3 Allow the beaker contents to stir for a minimum of 30 minutes before beginning enumeration.

11.3.4 While the stir bar is still spinning, remove a 10-μL aliquot and carefully load one side of the hemacytometer. Count all organisms on the platform, at 200X magnification using phase-contrast or darkfield microscopy. The count must include the entire area under the hemacytometer, not just the four outer 1-square-mm squares. Repeat this procedure nine times. This step allows confirmation of the number of oocysts per 10 μL (Section 11.2.12). If the mean number is outside the expected range, add additional oocysts from stock suspension or dilute the contents of the beaker appropriately with reagent water. Repeat the process to confirm counts. Refer to Section 11.2.14 for factors that may introduce errors.

11.4 Enumerating the oocyst spiking suspension using well slides

11.4.1 Remove 12-mm-diameter well slides from cold storage and lay the slides on a flat surface for 15 minutes to allow them to warm to room temperature.

11.4.2 Vortex the tube containing oocyst spiking suspension (diluted stock oocyst suspension; Section 11.2) for a minimum of 2 minutes. Gently invert the tube three times.

11.4.3 Remove a 10-μL aliquot from the oocyst spiking suspension tube and apply it to the center of a well.

11.4.4 Before removing subsequent aliquots, cap the tube and gently invert it three times to ensure oocysts are in suspension.

11.4.5 Ten wells must be counted, and the counts averaged, to sufficiently enumerate the spike dose.

11.4.6 Positive and negative controls must be prepared.

11.4.6.1 For the positive control, pipette 10 μL of positive antigen or 200 to 400 intact oocysts onto the center of a well and distribute positive antigen or oocysts evenly over the well area.

11.4.6.2 For the negative control, pipette 75 μL of PBS (Section 7.8.3) onto the center of a well and spread it over the well area with a pipette tip.

11.4.7 Place the well slides containing the samples in a 42°C incubator and evaporate to dryness (approximately 1 to 2 hours).

11.4.8 Apply 50 μL of absolute methanol to each well containing the dried sample and allow the slide to air dry until the methanol has evaporated (approximately 3 to 5 minutes).

11.4.9 Follow manufacturer's instructions in preparing dilutions of anti-*Cryptosporidium* fluorescein-labeled monoclonal antibody (Mab) and overlay the 10 spike-dose wells, the positive-control well and the negative-control well with 50 μL of fluorescein-labeled Mab. Place the slides in a humid chamber and incubate at 37°C for approximately 30

minutes. The humid chamber consists of a tightly sealed plastic container containing damp paper towels on which the slides are placed.

11.4.10 After 30 minutes, remove the slides from the humid chamber. Use a clean Pasteur pipette tip attached to a vacuum source to gently aspirate the excess fluorescein-labeled Mab from the side of each well. When performing this step, ensure that the vacuum source is at the absolute minimum (<2 in. Hg vac.) and ensure that the pipette tip does not scratch the well surface.

11.4.11 Apply 65 to 75 μL of PBS (Section 7.8.3) to each of the 12 wells and allow to stand for 1 to 5 minutes, then aspirate the excess PBS. When removing the excess PBS, ensure that the pipette tip does not scratch the well surface. Repeat this washing procedure two more times.

11.4.12 Apply a drop of reagent water to each well and allow to stand for approximately 1 minute, then aspirate the excess reagent water.

11.4.13 Place the slides containing the fluorescein-labeled Mab in a dry box and allow the slides to stand in the dark for approximately 1 hour at room temperature. The dry box consists of a tightly sealed plastic container with desiccant in the bottom. A paper towel must be placed over the desiccant.

11.4.14 Apply 10 μL of DABCO/glycerol mounting medium (Section 7.6) to the center of each well.

11.4.15 Place a 22 × 50 mm coverslip on each three-well microscope slide and gently depress the coverslip at the edges. Use a tissue to remove excess mounting fluid from the edges of the coverslip, then seal the edges of the coverslip onto the slide by using clear nail polish. Store in a dry box, in the dark, until ready for enumeration.

11.5 Procedure for spiking samples in the laboratory with enumerated oocyst spiking suspension

11.5.1 Arrange a bottom-dispensing 10-L carboy to gravity-feed a capsule filter so the outlet will feed self-priming centrifugal pump (Figure 3).

11.5.2 Place a large, sterile stir bar in the carboy. Fill the carboy with 10.0 L of reagent water (for initial precision and recovery [Section 9.4] and ongoing precision and recovery [Section 9.7] samples) or with the 10-L field sample (for matrix spike samples [Section 9.5]). Place the carboy on the stir plate. Turn the stirrer on so that the bar creates a vortex.

11.5.3 Vortex the tube containing the spiking suspension (Section 11.3 or 11.4) for a minimum of 2 minutes. Rinse a pipette tip with Tween-20, 0.01% once, then with the well-mixed spiking suspension a minimum of five times before pulling an aliquot to be used to spike the carboy.

11.5.4 Add the spiking suspension to the carboy, delivering the oocysts below the surface of the reagent water. Allow the spiking suspension to mix for approximately 1 minute in the carboy.

11.5.5 Turn on the pump and allow the flow rate to stabilize. Set flow at the rate designated for the filter under test. As the carboy is depleted, check the flow rate and adjust if necessary.

11.5.6 When the water level approaches the discharge port of the carboy, turn off the stirrer and tilt the carboy so that it is completely emptied. At that time, turn off the pump and add 1 L of reagent water to the carboy. Swirl the contents to rinse down the sides.

11.5.7 Turn on the pump. Allow the pump to pull all the water through the filter and turn off the pump.

12.0 Sample Filtration and Elution

12.1 A capsule filter is used to filter the 10-L water sample received by the laboratory, according to the procedures in Section 12.2. Alternate procedures may be used if the laboratory first demonstrates that the quality control acceptance criteria listed in Table 1 in Section 21.0 are met.

12.2 Capsule filtration (adapted from Reference 20.7)

12.2.1 Flow rate adjustment

12.2.1.1 Connect the sampling system, minus the capsule, to a carboy filled with reagent water (Figure 3).

12.2.1.2 Turn on the pump and adjust the flow rate to 2.0 L/min.

12.2.1.3 Allow 2 to 10 L of reagent water to flush the system. Adjust the pump speed as required during this period. Turn off the pump when the flow rate has been adjusted.

12.2.2 Install the capsule filter in the line, securing the inlet and outlet ends with the appropriate clamps/fittings.

12.2.3 Record the sample number, sample turbidity (if not provided with the field sample), and the name of analyst filtering the sample on a bench sheet and on the capsule filter.

12.2.4 Filtration

12.2.4.1 Connect the sampling system to the field carboy of sample water, or transfer the sample water to the laboratory carboy used in Section 12.2.1.1.

NOTE: If the field sample is transferred to a laboratory carboy, the laboratory carboy must be cleaned and disinfected before it is used with another field sample.

12.2.4.2 Place the drain end of the sampling system tubing into an empty graduated container with a capacity of 10 to 15 L, calibrated at 9.0, 9.5, 10.0, 10.5, and 11.0 L (Section 6.17). This container will be used to determine the sample volume filtered.

12.2.4.3 Allow the carboy discharge tube and capsule to fill with sample water. Vent residual air using the bleed valve/vent port. Turn on the pump to start water flowing through the filter. Verify that the flow rate is 2 L/min.

12.2.4.4 After the sample has passed through the filter, turn off the pump. Allow the pressure to decay until flow stops.

12.2.5 Disassembly

12.2.5.1 Disconnect the inlet end of the capsule filter assembly while maintaining the level of the inlet fitting above the level of the outlet fitting to prevent backwashing and the loss of oocysts from the filter. Restart the pump and allow as much water to drain as possible. Turn off the pump.

12.2.5.2 Based on the water level in the graduated container (Section 12.2.4.2), record the volume filtered on a bench sheet and the capsule filter label to the nearest quarter liter. Discard the contents of the graduated container.

12.2.5.3 Loosen the outlet fitting, then cap the inlet and outlet fittings.

12.2.6 Elution

12.2.6.1 Setup

12.2.6.1.1 Assemble the laboratory shaker with the clamps aligned vertically so that the filters will be aligned horizontally.

12.2.6.1.2 Prepare sufficient elution buffer so that all samples to be eluted that day can be eluted with the same batch of buffer. Elution may require up to 275 mL of buffer per sample.

12.2.6.1.3 Designate at least one 250-mL conical centrifuge tube for each sample and label with the sample number.

12.2.6.2 Elution

12.2.6.2.1 Record the name of the analyst performing the elution on a bench sheet. Using a ring stand or other means, clamp each capsule in a vertical position with the inlet end up. Remove the inlet cap and allow the liquid level to stabilize.

12.2.6.2.2 Pour elution buffer through the inlet fitting. Sufficient elution buffer must be added to cover the pleated white membrane with buffer solution. Replace the inlet cap and clamp the cap in place.

12.2.6.2.3 Securely clamp the capsule in one of the clamps on the laboratory shaker with the bleed valve positioned at the top on a vertical axis (in the 12 o'clock position). Turn on the shaker and set the speed to 80% of maximum (approximately 600 rpm). Agitate the capsule for approximately 5 minutes.

12.2.6.2.4 Remove the filter from the shaker, remove the inlet cap, and pour the contents of the capsule into the 250-mL conical centrifuge tube.

12.2.6.2.5 Clamp the capsule vertically with the inlet end up and add sufficient volume of elution buffer through the inlet fitting to cover the pleated membrane. Replace the inlet cap and clamp in place.

12.2.6.2.6 Return the capsule to its clamp on the shaker with the bleed valve positioned on a horizontal axis (3 or 9 o'clock position). Turn on the shaker and agitate the capsule for approximately 5 minutes. Add the contents of the capsule to the centrifuge tube.

12.2.7 Proceed to Section 13.0 for concentration and separation (purification).

13.0 Sample Concentration and Separation (Purification)

13.1 During concentration and separation, the filter eluate is concentrated through centrifugation, and oocysts in the sample are separated from other particulates through immunomagnetic separation (IMS). Alternate procedures and products may be used if the laboratory first demonstrates that the quality control acceptance criteria listed in Table 1 in Section 21.0 are met.

13.2 Adjustment of pellet volume

13.2.1 Centrifuge the 250-mL centrifuge tube containing the capsule filter eluate at 1100 × G for 15 minutes. Allow the centrifuge to coast to a stop. Record the initial pellet volume (volume of solids) and the date and time that concentration was completed on a bench sheet.

13.2.2 Using a Pasteur pipette, carefully aspirate off the supernatant to just above the pellet (or to 1 mL if the sample is reagent water). If the sample is reagent water (e.g. initial or ongoing precision and recovery sample) extra care must be taken to avoid aspirating oocysts during this step.

13.2.2.1 If the packed pellet volume is less than or equal to 0.5 mL, add reagent water to the centrifuge tube to bring the total volume to 10 mL. Vortex the tube for 10 to 15 seconds to resuspend the pellet. Proceed to Section 13.3.

13.2.2.2 If the packed pellet volume is greater than 0.5 mL, use the following formula to determine the total volume required in the centrifuge tube:

$$total\,volume\,(mL)\,required = \frac{pellet\,volume}{0.5\,mL} \times 10\,mL$$

(For example, if the packed pellet volume is 0.8 mL, the total volume required is 16 mL.) Add reagent water to the centrifuge tube to bring the total volume to the level calculated above. Vortex the tube for 10 to 15 seconds to resuspend the pellet. Record this resuspended volume on a bench sheet.

13.2.2.2.1 If the packed pellet volume is greater than 0.5 mL, *and only one slide will be prepared as a representative portion of the sample*, proceed to Section 13.3, and use a Pasteur pipette to transfer 10 mL of the resuspended sample (which will contain 0.5 mL of solids) to the flat-sided sample tube in Section 13.3.2.1.

13.2.2.2.2 If the packed pellet volume is greater than 0.5 mL, *and analysis of the entire sample is required:*

(a) Add additional reagent water to the centrifuge tube to bring the volume to an amount evenly divisible by 10, then vortex the tube for 10 to 15 seconds to resuspend. (For example, if the resuspended volume measured in Section 13.2.2.2 is 16 mL, add 4 mL of reagent water to bring the volume to 20 mL.) Record this final resuspended volume on a bench sheet.

(b) Proceed to Section 13.3, and process the sample as multiple, independent 10-mL subsamples from Section 13.3.2 onward, including the preparation and examination of separate slides for each aliquot.

13.3 IMS procedure (adapted from Reference 20.8)

13.3.1 Preparation of reagents

13.3.1.1 Prepare a 1X dilution of SL-buffer-A from the 10 X SL-buffer-A (clear, colorless solution) supplied. Use reagent water (demineralized; Section 7.3) as the diluent. For every 1 mL of 1X SL-buffer-A required, take 100

μL of 10 X SL-buffer-A and make up to 1 mL with the diluent water. A volume of 1.5 mL of 1X SL-buffer-A will be required per sample or subsample on which the Dynal IMS procedure is performed.

13.3.1.2 To a flat-sided sample tube (Section 6.4.4) add 1 mL of the 10 X SL-buffer-A (supplied—not the diluted 1X SL-buffer-A).

13.3.1.3 Add 1 mL of the 10 X SL-buffer-B (supplied—magenta solution) to the sample tube containing the 10 X SL-buffer-A.

13.3.2 Oocyst capture

13.3.2.1 Quantitatively transfer the water sample concentrate from Section 13.2.2 to the flat-sided sample tube containing the SL-buffer. Label the tube with the sample number.

13.3.2.2 Cap the tube and vortex the Dynabeads® anti-*Cryptosporidium* for approximately 10 seconds to suspend the beads. Ensure that the beads are fully resuspended by inverting the sample tube and making sure that there is no residual pellet at the bottom.

13.3.2.3 Add 100 μL of the resuspended beads (Section 13.3.2.2) to the sample tube containing the water sample concentrate and SL-buffer.

13.3.2.4 Affix the sample tube to a rotating mixer and rotate at approximately 25 rpm for 1 hour.

13.3.2.5 After rotating for 1 hour, remove the sample tube from mixer and place in the magnetic particle concentrator (MPC-1) with flat side of tube toward the magnet.

13.3.2.6 Without removing the sample tube from the MPC-1, place the magnet side of the MPC-1 downwards, so the tube is horizontal and the flat side of the tube is facing down.

13.3.2.7 Gently rock the sample tube by hand end-to-end through approximately 90°, tilting cap-end and base-end of the tube up and down in turn. Continue the tilting action for 2 minutes with approximately one tilt per second.

13.3.2.8 Ensure that the tilting action is continued throughout this period to prevent binding of low-mass, magnetic or magnetizable material. If the sample in the MPC-1 is allowed to stand motionless for more than 10 seconds, repeat Section 13.3.2.7 before continuing procedure.

13.3.2.9 Return the MPC-1 to the upright position, sample tube vertical, with cap at top. Immediately remove cap and pour off all of the supernatant from the tube held in the MPC-1 into a suitable container. Do not shake the tube and do not remove the tube from MPC-1 during this step.

13.3.2.10 Remove the sample tube from the MPC-1 and resuspend the sample in 1-mL 1X SL-buffer-A (prepared from 10X SL-buffer-A stock—supplied). Mix very gently to resuspend all material in the tube. Do not vortex.

13.3.2.11 Quantitatively transfer all the liquid from the sample tube to a labeled, 1.5-mL microcentrifuge tube.

13.3.2.12 Place the microcentrifuge tube into the second magnetic particle concentrator (MPC-M), with magnetic strip in place.

13.3.2.13 Without removing the microcentrifuge tube from MPC-M gently rock/roll the tube through 180° by hand. Continue for approximately 1 minute with approximately one 180° roll/rock per second. At the end of

this step, the beads and oocysts should produce a distinct brown dot on the back of the tube.

13.3.2.14 Immediately aspirate the supernatant from the sample tube and cap held in the MPC-M. If more than one sample is being processed, conduct three 180° rock/roll actions before removing the supernatant from each tube. Take care not to disturb the material attached to the wall of the tube adjacent to the magnet. Do not shake the tube. Do not remove tube from MPC-M while conducting these steps.

13.3.3 Dissociation of beads/oocyst complex

13.3.3.1 Remove the magnetic strip from the MPC-M.

13.3.3.2 Add 100 µL of 0.1 N HCl, then vortex vigorously for 10 to 15 seconds.

13.3.3.3 Incubate for 5 minutes at room temperature.

13.3.3.4 Vortex vigorously for 5 to 10 seconds

13.3.3.5 Replace magnetic strip in MPC-M and replace tube in MPC-M. Gently rock the tube end to end through approximately 90°, tilting cap-end and base-end of the tube up and down in turn. Continue the tilting action for 30 seconds with approximately one tilt per second.

13.3.3.6 Remove well slides from cold storage and lay the slides on a flat surface for 15 minutes to allow them to warm to room temperature.

13.3.3.7 Add 10 µL of 1.0 N NaOH to a sample well.

13.3.3.8 Do not remove the microcentrifuge tube from the MPC-M. Transfer all of the sample from the microcentrifuge tube in the MPC-M to the sample well with the NaOH. Do not disturb the beads at the back wall of the tube.

13.3.3.9 Proceed to Section 14.0 for sample staining unless a second dissociation is required. If a second dissociation is required (this may enhance recovery of oocysts in some cases), do not discard the beads or microcentrifuge tube that remain after transferring the sample to a well slide. Perform the steps in Sections 13.3.3.1 through 13.3.3.8 a second time. Apply the resulting volume collected in Section 13.3.3.8 to a second sample well and proceed to Section 14.0 for sample staining.

14.0 Sample Staining

14.1 Prepare positive and negative controls. For the positive control, pipette 10 µL of positive antigen or 200 to 400 intact oocysts to the center of a well. For the negative control, pipette 75 µL of PBS (Section 7.8.3) into the center of a well and spread it over the well area with a pipette tip.

14.2 Place the well slides containing the samples in a 42°C incubator and evaporate to dryness (approximately 1 to 2 hours).

14.3 Apply 50-µL of absolute methanol to each well containing the dried sample and allow to air dry for 3 to 5 minutes.

14.4 Follow manufacturer's instructions in preparing dilutions of anti-*Cryptosporidium* fluorescein-labeled monoclonal antibody (Mab) and overlay the sample well, the positive-control well, and the negative-control well with 50 µL of fluorescein-labeled Mab. Place the slides in a humid chamber and incubate at 37°C for approximately 30 minutes. The humid chamber consists of a tightly sealed plastic container containing damp paper towels on which the slides are placed.

14.5 After 30 minutes, remove the slides and use a clean Pasteur pipette tip attached to a vacuum source to gently aspirate excess fluorescein-labeled Mab from the side of each well. When

performing this step, ensure that the vacuum source is at a minimum (<2 in. Hg vac.) and ensure that the pipette tip does not scratch the well surface.

14.6 Apply 65 to 75 μL of PBS (Section 7.8.3), to each well and allow to stand for 1 to 5 minutes, then aspirate the excess PBS. When removing the excess PBS, ensure that the pipette tip does not scratch the well surface. Repeat this washing procedure two more times.

14.7 Apply 50 μL of 4',6-diamidino-2-phenylindole (DAPI) staining solution (Section 7.7.2.2) to each well. Allow to stand at room temperature for approximately 2 minutes.

14.8 Remove the excess DAPI solution by aspiration.

14.9 Apply 65 to 75 μL of PBS (Section 7.8.3) to each well and allow to stand for 1 to 5 minutes, then aspirate the excess PBS. When removing the excess PBS, ensure that the pipette tip does not scratch the well surface. Repeat this washing procedure two more times.

14.10 Apply 65 to 75 μL of reagent water to each well and allow to stand for approximately 1 minute, then aspirate the excess reagent water.

14.11 Place the slide in a dry box and allow the slides to stand in the dark for approximately 1 hour at room temperature. The dry box consists of a tightly sealed plastic container with desiccant in the bottom. A paper towel must be placed over the desiccant.

14.12 Apply 10 μL of DABCO/glycerol mounting medium (Section 7.6) to the center of each well.

14.13 Place a 22 × 50 mm coverslip on each three-well microscope slide and gently depress the coverslip at the edges. Use a tissue to remove excess mounting fluid from the edges of the coverslip and then seal the edges of the coverslip onto the slide using clear nail polish. Record the date and time that staining was completed on a bench sheet. Store slides in a dry box, in the dark, until ready for examination.

15.0 Examination

15.1 Scanning technique: Scan each well in a systematic fashion. An up-and-down or a side-to-side scanning pattern may be used (Figure 4).

15.2 Examination and confirmation using immunofluorescence assay (FA), 4',6-diamidino-2-phenylindole (DAPI) staining characteristics, and differential interference contrast (D.I.C.) microscopy. Record examination and confirmation results on a *Cryptosporidium* report form. All confirmed oocysts must be reported.

15.2.1 If the positive staining control contains oocysts within the expected range and at the appropriate fluorescence for both FA and DAPI, and the negative staining control does not contain any oocysts (Section 14.1), use epifluorescence to scan the entire well for each sample at not less than 200X total magnification for apple-green fluorescence of oocyst shapes.

15.2.2 When brilliant apple-green fluorescing ovoid or spherical objects 4 to 6 μm in diameter are observed with brightly highlighted edges, switch the microscope to the UV filter block for DAPI, then to D.I.C.

15.2.2.1 Using the UV filter block for DAPI, the object will exhibit one of the following characteristics:

(a) Up to four distinct, sky-blue nuclei
(b) Intense blue internal staining
(c) Light blue internal staining (no distinct nuclei)

(a) and (b) are recorded as DAPI +; (c) is recorded as DAPI -.

15.2.2.2 Using D.I.C., look for external or internal morphological characteristics atypical of *Cryptosporidium* oocysts (e.g., spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.) (adapted from Reference 20.6).

15.2.2.2.1 If atypical structures are not observed, then categorize each apple-green fluorescing object as:

(a) An empty *Cryptosporidium* oocyst

(b) A *Cryptosporidium* oocyst with amorphous structure

(c) A *Cryptosporidium* oocyst with internal structure (one to four sporozoites/oocyst)

Record the shape and measurements to the nearest 0.5 μm at 1000X total magnification for each such object. Although not a defining characteristic, surface oocyst folds may be observed in some specimens.

15.2.2.2.2 For each oocyst, record the number of sporozoites observed. *Cryptosporidium* oocysts with sporozoites must be confirmed by a principal analyst/supervisor. Record the date and time that sample examination and confirmation was completed on the report form.

16.0 Analysis of Complex Samples

16.1 Some samples may contain high levels (>1000/L) of oocysts and/or interfering organisms, substances, or materials. Some samples may clog the filter (Section 12.0); others will not allow separation of the oocysts from the retentate or eluate; and others may contain materials that preclude or confuse microscopic examination. In these cases, dilute the original sample and filter and analyze 10 L of the diluted sample. If the sample is diluted at any step during analysis, the laboratory must record the original and final volumes and the volume analyzed.

16.2 If the sample holding time has not been exceeded and a full 10-L sample cannot be filtered, dilute an aliquot of sample to 10 L with reagent water and filter this smaller aliquot (Section 12.0). This dilution must be recorded and reported with the results.

16.3 If the holding times for the sample and for microscopic examination of the cleaned up retentate/eluate have been exceeded, the site must be re-sampled.

17.0 Method Performance

17.1 Required method performance data are shown in Table 1 in Section 21.0. These data are based on an interlaboratory validation study of Method 1622 involving 12 laboratories and 12 raw surface water matrices across the U.S.

18.0 Pollution Prevention

18.1 The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.

18.2 Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

19.0 Waste Management

19.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the biohazard and hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required. An overview of these requirements can be found in the *Environmental Management Guide for Small Laboratories* (EPA 233-B-98-001).

19.2 Samples, reference materials, and equipment known or suspected to have viable oocysts attached or contained must be sterilized prior to disposal.

19.3 For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel* and *Less is Better: Laboratory Chemical Management for Waste Reduction*, both available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington, D.C. 20036.

20.0 References

20.1 Rodgers, Mark R., Flanigan, Debbie J., and Jakubowski, Walter, *Applied and Environmental Microbiology* 61(10): 3759-3763 (October 1995).

20.2 Fleming, Diane O., et al. (eds.), *Laboratory Safety: Principles and Practices*, 2nd edition. 1995. ASM Press, Washington, DC

20.3 "Working with Carcinogens," DHEW, PHS, CDC, NIOSH, Publication 77-206, (Aug 1977).

20.4 "OSHA Safety and Health Standards, General Industry," OSHA 2206, 29 *CFR* 1910 (Jan 1976).

20.5 "Safety in Academic Chemistry Laboratories," ACS Committee on Chemical Safety (1979).

20.6 *ICR Microbial Laboratory Manual*, EPA/600/R-95/178, National Exposure Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, 26 Martin Luther King Drive, Cincinnati, OH 45268.

20.7 "Envirochek™ Sampling Capsule," PN 32915, Gelman Sciences, 600 South Wagner Road, Ann Arbor, MI 48103-9019 (September 1996).

20.8 "Dynabeads® anti-Cryptosporidium Prototype Procedure, Second Revision," Dynal Microbiology R&D, P.O. Box 8146 Dep., 0212 Oslo, Norway (May 1997).

21.0 Tables and Figures

NOTE: The acceptance criteria listed in Table 1 are based on data generated through interlaboratory validation of Method 1622 involving 12 laboratories and 12 raw surface water matrices.

Table 1. Quality control acceptance criteria for performance tests for *Cryptosporidium*

Performance test	Section	Acceptance criteria
Initial precision and recovery (IPR)	9.4	
Mean recovery (percent)	9.4.2	19 - 100
Precision (as maximum relative standard deviation)	9.4.2	54
Ongoing precision and recovery (OPR) (percent)	9.7	10 - 100
Matrix spike/matrix spike duplicate (MS/MSD)	9.5	
Mean recovery* (as percent)	9.5	13 - 143
Precision (as maximum relative percent difference)	9.5	67

*The acceptance criteria for mean MS/MSD recovery serves as the acceptance criteria for MS recovery during routine use of the method (Section 9.5.1)

Figure 1. Hemacytometer platform ruling. Squares 1, 2, 3, and 4 are used to count stock suspensions of *Cryptosporidium* oocysts (after Miale, 1967)

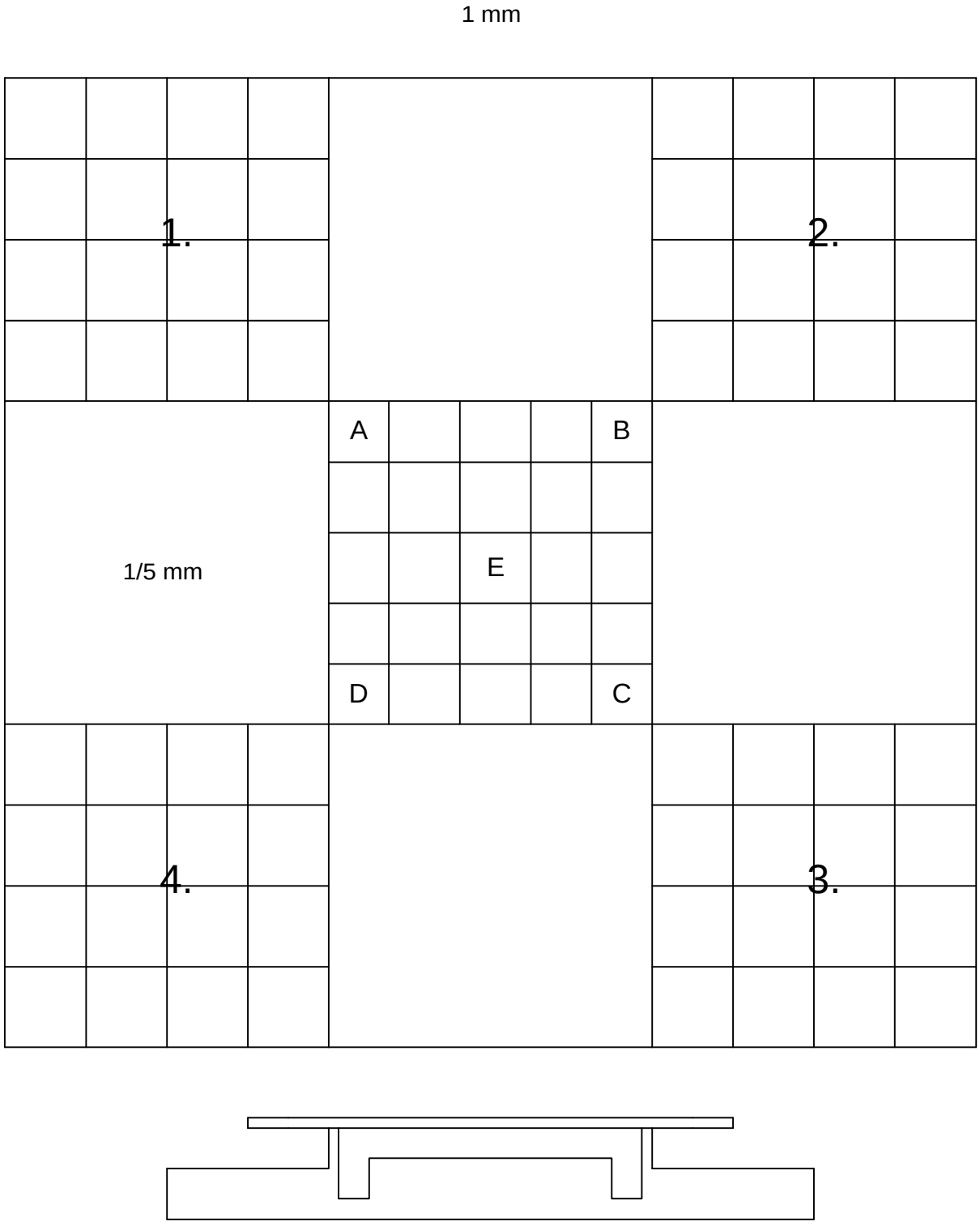


Figure 2. Manner of counting *Cryptosporidium* oocysts in 1 square mm. Dark oocysts are counted and light oocysts are omitted (after Miale, 1967)

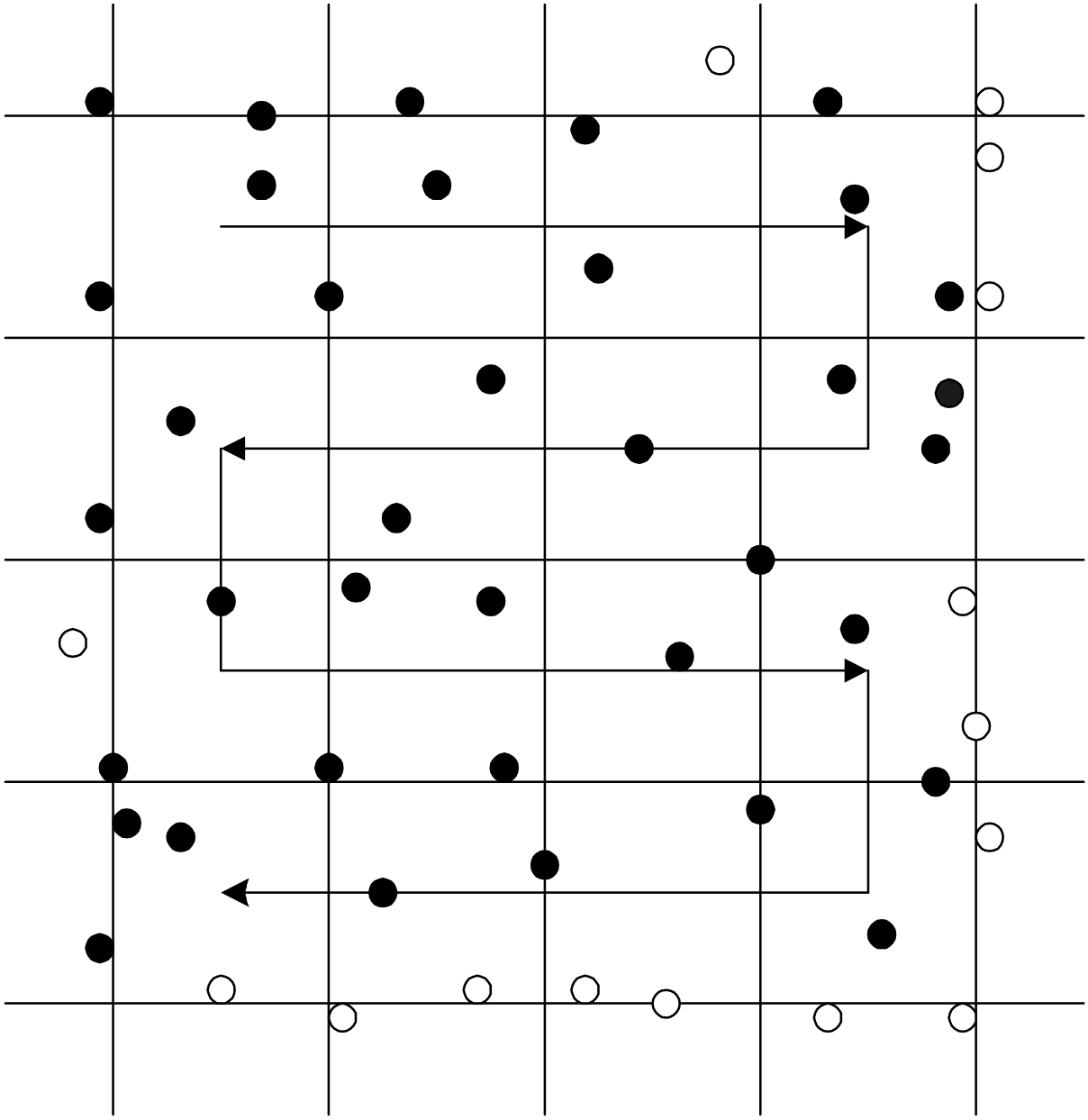


Figure 3. Laboratory filtration system

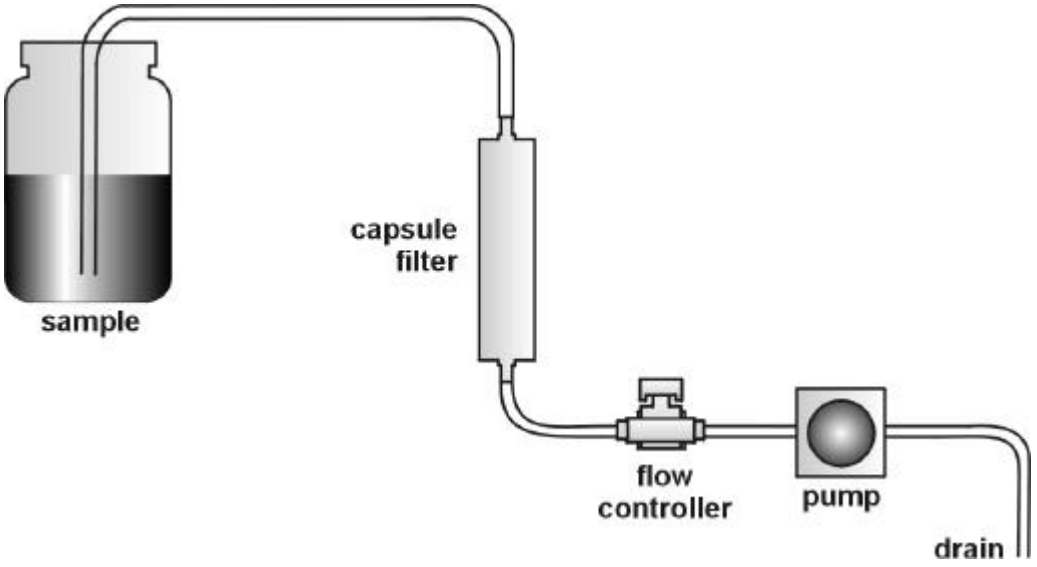
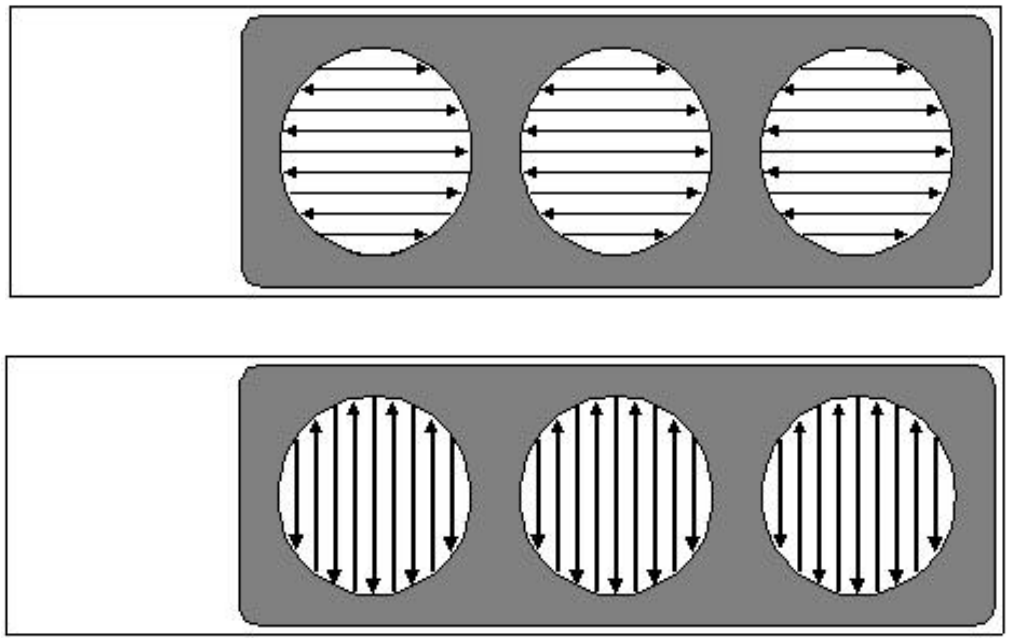


Figure 4. Methods for scanning a well slide



22.0 Glossary of Definitions and Purposes

These definitions and purposes are specific to this method but have been conformed to common usage as much as possible.

22.1 Units of weight and measure and their abbreviations

22.1.1 Symbols

°C	degrees Celsius
μL	microliter
<	less than
>	greater than
%	percent

22.1.2 Alphabetical characters

cm	centimeter
g	gram
G	acceleration due to gravity
hr	hour
ID	inside diameter
in.	inch
m	meter
mg	milligram
mL	milliliter
mm	millimeter
mM	millimolar
N	normal; gram molecular weight of solute divided by hydrogen equivalent of solute, per liter of solution
s_r	standard deviation of recovery
X	average percent recovery

22.2 Definitions, acronyms, and abbreviations (in alphabetical order)

Analyst—The analyst must have 2 years of college lecture and laboratory course work in microbiology or a closely related field. The analyst also must have at least 6 months bench experience, must have at least 3 months experience with FA techniques, and must have successfully analyzed at least 50 water and/or wastewater samples for *Cryptosporidium*. Six months of additional experience in the above areas may be substituted for two years of college.

Analyte—A protozoan parasite tested for by this method. The analyte in this method is *Cryptosporidium*.

Immunomagnetic separation (IMS)—A purification method that uses microscopic, magnetically responsive particles coated with an antibodies targeted to react with a specific pathogen in a fluid stream. Pathogens are selectively removed from other debris using a magnetic field.

Initial precision and recovery (IPR)—four aliquots of oocyst spiking suspension analyzed to establish the ability to generate acceptable precision and accuracy. An IPR is performed prior to the first time this method is used and any time the method or instrumentation is modified.

Laboratory blank—See Method blank

Laboratory control sample (LCS)—See Ongoing precision and recovery (OPR) standard

Matrix spike (MS)—A sample prepared by adding a known quantity of organisms to a specified amount of sample matrix for which an independent estimate of target analyte concentration is available. A matrix spike is used to determine the effect of the matrix on a method's recovery efficiency.

May—This action, activity, or procedural step is neither required nor prohibited.

May not—This action, activity, or procedural step is prohibited.

Method blank—An aliquot of reagent water that is treated exactly as a sample including exposure to all glassware, equipment, solvents, and procedures that are used with samples. The method blank is used to determine if analytes or interferences are present in the laboratory environment, the reagents, or the apparatus.

Must—This action, activity, or procedural step is required.

Negative control—See Method blank

Nucleus—A membrane-bound organelle containing genetic material. Nuclei are a prominent internal structure seen in *Cryptosporidium* oocysts. In *Cryptosporidium* oocysts, there is one nucleus per sporozoite.

Oocyst—The encysted zygote of some sporozoa; e.g., *Cryptosporidium*. The oocyst is a phase or form of the organism produced as a normal part of the life cycle of the organism. It is characterized by a thick and environmentally resistant outer wall.

Ongoing precision and recovery (OPR) standard—A method blank spiked with known quantities of analytes. The OPR is analyzed exactly like a sample. Its purpose is to assure that the results produced by the laboratory remain within the limits specified in this method for precision and recovery.

Oocyst spiking suspension—See Spiking suspension

Oocyst stock suspension—See Stock suspension

Positive control—See Ongoing precision and recovery standard

Principal analyst/supervisor—The principal analyst/supervisor must be an experienced microbiologist with at least a B.A./B.S. in microbiology or a closely related field. The principal analyst also must have at least 1 year of continuous bench experience with immunofluorescent antibody (FA) techniques and microscopic identification and have analyzed at least 100 water and/or wastewater samples for *Cryptosporidium*.

PTFE—Polytetrafluoroethylene

Quantitative transfer—The process of transferring a solution from one container to another using a pipette in which as much solution as possible is transferred, followed by rinsing of the walls of the source container with a small volume of rinsing solution (e.g., reagent water, buffer, etc.), followed by transfer of the rinsing solution, followed by a second rinse and transfer.

Reagent water—Water demonstrated to be free from the analytes of interest and potentially interfering substances at the method detection limit for the analyte.

Reagent water blank—see Method blank

Relative standard deviation (RSD)—The standard deviation times 100 divided by the mean.

RSD—See Relative standard deviation

Should—This action, activity, or procedural step is suggested but not required.

Spiking suspension—Diluted stock suspension containing the organism(s) of interest at a concentration appropriate for spiking samples.

Sporozoite—A motile, infective stage of certain protozoans; e.g., *Cryptosporidium*. There are four sporozoites in each *Cryptosporidium* oocyst, and they are generally banana-shaped.

Stock suspension—A concentrated suspension containing the organism(s) of interest that is obtained from a source that will attest to the host source, purity, authenticity, and viability of the organism(s).

Technician—The technician filters samples, performs centrifugation, elution, concentration, and purification using IMS, and places purified samples on slides for microscopic examination, but does not perform microscopic protozoan detection and identification. The technician must have at least 3 months of experience in filter extraction and processing of protozoa samples.